Evolutionary genetics of eelgrass clones in the Baltic Sea

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草の名も　治定しにけり　春の雨

Frühlingsregen fällt,

und alles, was da grünt, hat

plötzlich seinen Namen

Haiku von Komatomi, Osaka 1754

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CONTENTS

SUMMARY

Seagrasses are a group of marine flowering plants thriving in shallow coastal waters worldwide. The study species of this thesis, eelgrass (*Zostera marina*), is the dominant seagrass species of the northern temperate zone. As most other seagrass species, eelgrass shows extensive clonal growth and in the non tidal Baltic Sea its clones can persist over many years. During growth, eelgrass clones become fragmented at several spatial scales because the root connections between ramets (rhizome units) break over time. This fragmentation prohibits clone identification in the field, in particular in dense meadows. However, as clones are genetic individuals and hence ultimately subject to selection, they represent the relevant level to address questions in an evolutionary context.

In this thesis I studied the clonal structure in dense eelgrass meadows in the Baltic Sea in the context of the mating system, inbreeding depression, local adaptation, kinship structure and genet dynamics. I used microsatellite markers to access the fine scale (1-m) clonal structure in four 15-m x 15-m plots located within dense eelgrass meadows in two populations on the Baltic Coast (1.5 – 3.5 m water depth). These plots served as templates for the selection of replicated transplants for laboratory and field experiments, as permanently marked areas for resampling and tracking genets, and as database for the calculation of genetic parameters. The key questions investigated and their answers were the following: (i) Can flowering ramets recognize their genetic neighbourhood through pollen and/or growth interactions? - Addition of self versus cross pollen affected the inflorescence sex ratio. This can only be explained by the presence of a cryptic self-incompatibility system. Such self-recognition may reduce fitness costs through inbreeding. (ii) Does inbreeding depression influence the size distribution of eelgrass clones? - The level of heterozygosity was higher in larger clones. Together with measures of reproductive output this suggests that large dominant clones outcompete their relatively inbred neighbours in an environment with low levels of disturbance. (iii) Are eelgrass clones locally adapted? - Transplantation of replicated genets between two populations showed significant local adaptation across both sites and dominance of genets at one site. (iv) Does limited gene flow lead to kinship structure beyond the spatial spread of eelgrass clones? - Spatial autocorrelation, modified for a clonal species, revealed significant coancestry (f_{ij}) for the neighbourhood of ramets, clone fragments and entire clones. (v) What are the demographic parameters of eelgrass genets? - Eelgrass clones showed surprisingly high turnover across genets but almost constant patterns within genets between the years 2000 and 2001. This suggests a genetic component to flowering intensity and the production of vegetative shoots. This last study was designed to continue for

several more years.

In conclusion, the clonal patterns and the mating landscape in eelgrass meadows are profoundly influenced by limited seed and pollen dispersal, and by selection for outbred clones. If cryptic self-incompatibility is adaptive, then eelgrass individuals may be well equipped to buffer negative effects of the changing geometry in their genetic neighbourhood on sexual reproduction. The geometry of the genetic neighbourhood is changing surprisingly fast. Within clones however, patterns of reproductive output remain constant over time. Such an evolutionary view into eelgrass genetics does also have implications in a conservation and restoration context. Genetic erosion, the loss of genetic diversity in an eelgrass meadow will ultimately lead to increased homozygosity. In such a scenario, selection for outbred clones and delayed selfing will cease. The depletion of genotypes will also increase kinship structure and hence the negative effects of biparental inbreeding. The import of new genetic material from foreign sites through long range dispersal is unlikely to be sufficient in order to stabilize the process of genetic erosion because most dispersal seems to be limited to only a few meters. Finally the selection of suitable transplants in eelgrass restoration can benefit from a combination of genetic marker data and phenotypic measurements to consider both genetic diversity and the degree of local adaptation in management decisions.

ZUSAMMENFASSUNG

Seegräser sind marine Blütenpflanzen, die weltweit in flachen Küstengewässern gedeihen. Modellpflanze dieser Arbeit ist das große Seegras, *Zostera marina*, die vorherrschende Seegrasart der Nordhemisphäre. Wie bei den meisten anderen Seegrasarten findet man auch bei *Zostera marina* neben sexueller Fortpflanzung über Samen ausgeprägtes klonales Wachstum. In der fast gezeitenlosen Ostsee können einzelne Klone dieser Art viele Jahre überdauern. Während Klone wachsen, werden sie zunehmend räumlich fragmentiert, weil die Wurzelverbindungen zwischen den Rameten (Rhizomeinheiten) mit der Zeit unterbrochen werden. Diese Fragmentierung verhindert, in der geschlossenen Wiese einzelne Seegras Klone *in situ* zu identifizieren. Weil aber die Klone und nicht die einzelnen Rameten die genetischen Individuen sind und deshalb direkt unter Selektion stehen, muss diese Betrachtungsebene im Mittelpunkt von evolutionsbiologischen Untersuchungen stehen.

In dieser Dissertation habe ich die Klonstruktur von *Zostera marina* im Zusammenhang mit dem Fortpflanzungssystem, möglicher Inzuchtdepression, lokaler Standortanpassung, der kleinräumigen Verwandtschaftsstruktur und der Dynamik von Geneten in dichten Seegraswiesen an der Ostseeküste untersucht. Mit Hilfe von Mikrosatelliten-Markern habe ich zunächst die Klonstruktur in vier 15m x 15m Flächen aus zwei Populationen in einem 1m Raster kartiert. Diese Flächen waren der Ausgangspunkt für die Auswahl von replizierten Verpflanzungseinheiten für Labor- und Feldexperimente. Sie fungierten gleichzeitig als Dauerbeobachtungsflächen für die Wiederbeprobung und als Datenbasis für die Berechnung von genetischen Parametern. Die untersuchten Schlüsselfragen dieser Arbeit und ihre Antworten sind die folgenden: (i) Können blühende Seegrasrameten ihre genetische Nachbarschaft durch Pollen und/oder Rhizom-Kontakte erkennen? - Die Zugabe von eigen- und ausgekreuzten Pollen beeinflusste das Geschlechterverhältnis von Seegrasähren in einem Mesokosmenexperiment. Dies kann nur mit dem Vorhandensein eines Inkompatibilitäts-Systems erklärt werden. Die Erkennung von Eigenpollen sollte Fitnessverluste durch Einkreuzung verringern. (ii) Hat Inzuchdepression einen Einfluss auf die Größenverteilung von Seegrasgeneten? - Grosse Klone hatten einen höheren Heterozygotiegrad. Zusammen mit Messungen zur reproduktiven Leistung deuten diese Resultate darauf hin, dass große Klone unter geringem Störungsregime ihre ingezüchteten Nachbarn verdrängen können. (iii) Sind Seegrasgeneten an ihre lokalen Standortbedingungen angepasst? - Verpflanzte Einheiten von replizierten Seegrasgeneten zeigten lokale Adaptation zwischen zwei Populationen. (iv) Führt räumlich limitierter Genfluss auf der untersuchten Skala zur Verwandtschaftsstruktur zwischen Seegras-Geneten? -

Räumliche Autokorrelation, welche für eine klonale Pflanze angepasst wurde, zeigte eine signifikante Verwandtschaftsstruktur (f_{ij}) für Rameten, Klonfragmente und Klone, die drei Ebenen der Klonstruktur. (v) Wie sieht die Demographie von Seegras-Klonen aus? - Die Dynamik zwischen Seegrasgeneten in zwei aufeinanderfolgenden Jahren war erstaunlich hoch, die Muster waren jedoch für individuelle Geneten relativ konstant. Dies deutet daraufhin, dass die Blühintensität und Produktion von vegetativen Sprossen eine starke genetische Komponente aufweisen. Diese letzte Studie ist so angelegt, dass sie noch einige Jahre weitergeführt werden kann.

Zusammenfassend ergibt sich folgendes Bild: Die Klonstruktur und die daraus resultierende Fortpflanzungs-Landschaft werden durch kurze Verbreitungsdistanzen von Samen und Pollen, sowie durch die positive Selektion von ausgekreuzten Klonen grundlegend beeinflusst. Sollte die identifizierte kryptische Selbstinkompatibilität adaptiv sein, so könnten Seegrasindividuen gut ausgerüstet sein, um mögliche negative Effekte in einer sich zeitlich ändernden Geometrie der genetischen Nachbarschaft zu verringern. Diese Geometrie scheint sich überraschend schnell zu ändern, während innerhalb der Geneten die Muster der reproduktiven Investition relativ konstant bleiben.

Eine evolutionsbiologische Sicht der Seegras Populationsbiologie ist auch für Schutzmassnahmen und Wiederansiedlung relevant. Genetische Erosion, der Verlust von genetischer Diversität in einer Seegraswiese führt zu erhöhter Homozygotie. Dieser Prozess wiederum wird die Selektion von ausgekreuzten Klonen und verzögerte Selbstbefruchtung zum Erliegen bringen. Eine mögliche Verarmung an Genotypen verstärkt zudem die Verwandtschaftsstruktur und damit die negativen Effekte von Inzucht. Der Import von neuem genetischen Material von außen wird sehr wahrscheinlich nicht genügen, um den Prozess der genetischen Erosion zu stoppen oder zumindest zu stabilisieren, da der überwiegende Anteil von Samen und Pollen nur kurze Distanzen zurücklegt. Die Auswahl von geeignetem Pflanzenmaterial für Wiederansiedlungsmaßnahmen schließlich profitiert von einer Kombination aus genetischen Markerdaten und phänotypischen Daten, um sowohl genetische Diversität als auch lokale Adaptation berücksichtigen zu können.

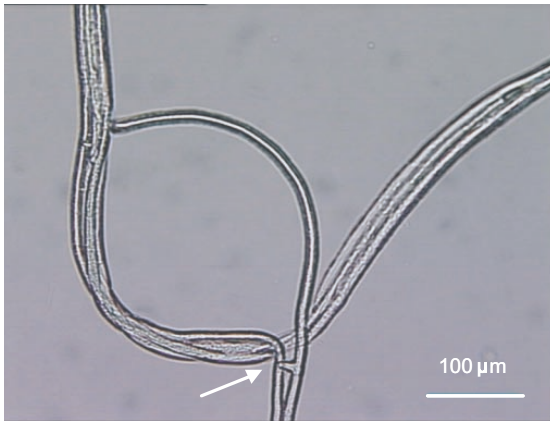

Introduction

In this general introduction I will present the necessary methodological tools and discuss the concepts of my research questions, which together formed the foundation for this thesis. All the studies described in chapters I – V were based on high resolution, fine scale genetic mapping of eelgrass (*Zostera marina*) clones on the Baltic Coast. The general denominator for all raised questions was how the clonal structure influenced the evolutionary dynamics of eelgrass meadows.

1 - SEAGRASSES: NOT MERELY A GREEN FRILL ON THE SHORELINE

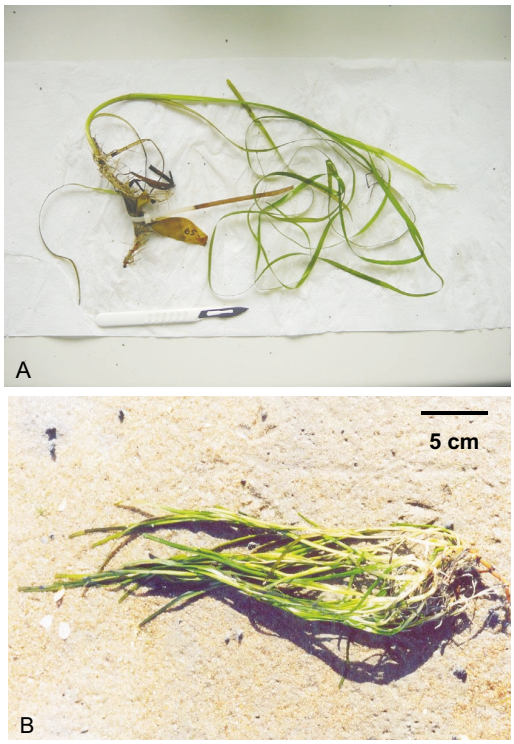
Seagrasses are a polyphyletic group of 49 species of marine flowering plants, which successfully dominate shallow coastal waters worldwide, except for the most polar regions (Den Hartog 1970). The name seagrass comes from their morphological resemblance with terrestrial grass species but botanically they are classified within the monocotyledonous plants (Heywood 1996). Although totally submerged, seagrasses have all the structures of their terrestrial relatives: a root system, a vascular system, sexual reproduction and most of them show extensive clonal growth. What makes this group of marine plants unique is that flowers are usually fertilized by water borne pollen (except *Enhalus acoroides*) (de Cock 1980; Rasmussen et al. 1973). Many seagrass species form dense monospecific stands, which can consist of up to 4000 shoots per m^2. Associated with this is an increase in “bottom” area by 15 to 20 times as a result of the leaf surface (Rasmussen et al. 1973). Hence, seagrass meadows provide abundant refuge and habitat for a diversity of animal species including juvenile lifestages of many commercially important fish (Jackson et al. 2001). Other important ecosystem functions are the filtering of seawater, removal of nutrients and stabilization of the sediment (Tomlinson 1974). On a global scale, seagrass meadows are amongst the most productive ecosystems. In terms of their economic value they have been put on the third place only after estuaries and

Zostera marina: Upper part of a flowering shoot (spadix) ready to bloom. The arrow indicates two already receptive stigmata (female flowers).

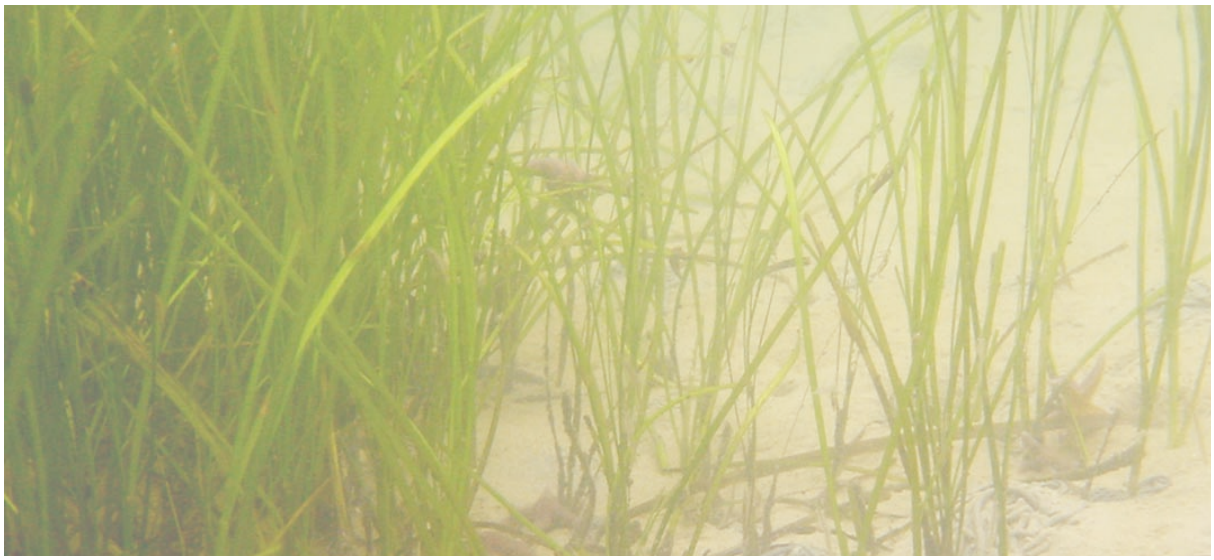


Threads of several encoiled *Zostera marina* pollen. The protuberation indicated with an arrow is the beginning of pollen tube growth.

floodplains (Costanza et al. 1997). In the past decades the anthropogenic impact along coastal zones has increased and it has been demonstrated that this increase is often linked to seagrass loss because of a decrease in water quality (Dennison et al. 1993). Apart from water pollution other yet largely unknown factors may have been responsible for the large-scale decline that was observed in many areas (Den Hartog 1996; Orth & Moore 1983b; Rasmussen 1973; Short & Wyllie-Escheverria 1996). Ironically, the disappearance of seagrass meadows has proved their importance and has promoted studies on restoration (Lent & Verschuure 1995; Orth Robert et al. 1999; Pranovi et al. 2000; Van Katwijk et al. 1998; Williams & Davis 1996; Worm & Reusch 2000; reviewed in Fonseca et al. 1988) and genetic diversity (Procaccini & Mazzella 1998; Reusch et al. 1999b; Ruckelshaus 1998; Waycott 1998). More recently seagrass meadows have also been recognized as an excellent study system for the biology of clonal plants and in particular their breeding system (Reusch 2000; Reusch 2001a; Ruckelshaus 1995; Ruckelshaus 1996) because in relatively undisturbed areas seagrasses still form large monospecific meadows through clonal growth unparalleled in



Physiologically independent rhizome units, as they can be found in eelgrass meadows. One unit can have (A) a few (Baltic Sea) to (B) several (North Sea) vegetative shoots.


Inshore margin of dense eelgrass meadow in Falkenstein (Kiel)

their extent in terrestrial systems.

The dominant seagrass species of the northern hemisphere and the study species of this work is eelgrass (*Zostera marina* L.). Eelgrass can form dense meadows, which is especially pronounced in the non-tidal Baltic Sea (in 2 - 6 m water depth) where it is also perennial. While open space is usually colonized through the clonal expansion of rhizomes, sexual reproduction and the recruitment of seedlings is contributing to an unquantified degree to the maintenance of population structure. Flowering shoots are produced once a year and can be easily distinguished from vegetative shoots in an early stage of development. Flowering shoots start to grow in winter and early spring, before the vegetative shoots and hence they stand out above the main canopy. In addition, flowering shoots have a somewhat woody structure at the base of the stems with a round profile. Flowering and seed ripening takes place during the summer months (de Cock 1980; Den Hartog 1970). Flowering shoots are branched with up to 14 inflorescences, each bearing several female and male flowers. The flowering phenology is protogynous at the level of single inflorescences (spadix). Among flowering shoots of the same clone (identical genotype) however, style erection and pollen release both occur simultaneously and hence, *Zostera marina* clones are functional hermaphrodites. Hand pollination has shown that *Z. marina* is self compatible (Ruckelshaus 1995). The filamentous morphology of the pollen has been interpreted as an adaptive trait for sub-aqueous pollination because their threads tend to coil themselves around obstacles once released (Ackerman 1997; Cox 1983). Although seed production is abundant, in closed, well established meadows, seedling recruitment and establishment is rare (Olesen & Sand-Jensen 1994b; Olesen & Sand-Jensen 1994c; Robertson & Mann 1984; & pers. obs.). Disturbance events are likely to play an important role by creating larger gaps that can be colonized through seedlings.

It is probably fair to say that eelgrass is the most extensively studied seagrass species to date. Apart from the scientific interest however, there has also been a long and ongoing tradition of using eelgrass in every day life. In previous times, people along the European Coast as well as the colonists in the New World used estuarine grasses as mattress filling, bedding for domestic animals, cattle forage, insulation, fertilizer, mulch and fuel (Heywood 1996). Even in areas where access to eelgrass was limited, the plant was known for its useful properties. For example people in the alpine precincts often cultivated and harvested the sedge *Carex brizoides* as eelgrass substitute in mattress fillings, which led to the name of seagrass beech forest for remnants of this vegetation type (Ellenberg 1996). Still today, the seeds of eelgrass are harvested from the Gulf of California by the Seri Indians and used to prepare gruel (www.fws.gov). And, very recently, eelgrass has been discovered for its bal-

anced concentration of mineral and trace elements and is used for wellness applications developed for example by AQUAZOSTA ® (www.manetmail.de). The importance of eelgrass and seagrasses in general pointed out above and the list of applications, which is by no means exhaustive, should make clear that ‘seagrasses are not merely a green frill on the shoreline’ (Rasmussen et al. 1973) but rather protagonists in the fascinating ecosystem of marine coastal waters.

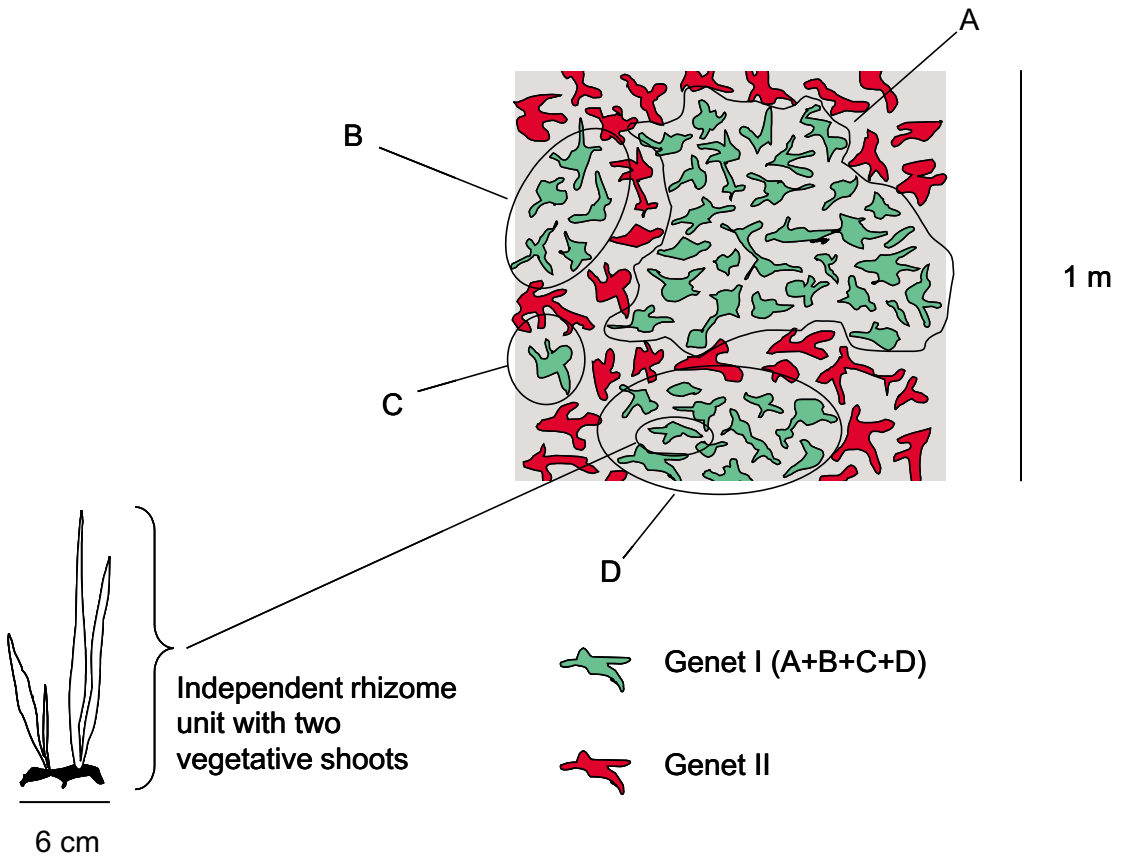
2 - WHAT ARE EELGRASS INDIVIDUALS ? - THE DANDELION CONCEPT

Seventy – 80 % of plant species in the temperate zone are clonal plants (Groenendael & Kroon 1990; Klimes et al. 1997). Clonal plants often successfully dominate aquatic and terrestrial vegetation and some species are known for their invasive colonization pattern (Harper 1981; Schmid 1990). Most seagrass species including eelgrass also have a clonal growth form (Den Hartog 1970), which may be a prerequisite to persist in the coastal environment under the influence of strong mechanical forces by waves and currents. The geometry of clonal growth may have severe consequences for sexual reproduction and population dynamics (Handel 1985) and it is a key issue in the present thesis. For this reason I want to discuss the patterns of clonal growth in more detail in the following paragraph.

Common for all clonal plants is that they grow through the reiteration of modular units. These units can be breeding buds as in the potato, rhizome splitters as in eelgrass or even apomictic (genetically identical) seeds as in dandelion (*Taraxacum sp*.). Each shoot or shoot bundle emerging from a single such unit could be (and has often been) looked at as an individual plant. This seems straightforward and is what we find in the field. However, ultimately selection acts at the level of the genetic individual (Maynard Smith 1976) and hence, an alternative view is to look at the sum of all such modular units which share a common genotype as one individual. These two alternative views are not compatible because they strongly influence the life history parameters measured for a particular plant species in a given area. Janzen (1977) called this dualism the “confusion in dandelion ecology”. With the first view in mind, we would look at a many-membered population with very high growth rate and short-lived dandelion plants. In contrast, with the second view, the dandelion population would consist of a small number of highly fragmented individuals with long lives and very low population growth rate. And the same logic applies to eelgrass. Instead of thousands of shoot-individuals per m^2, eelgrass consists of much fewer genetic individuals (clones) some of which can cover extensive areas. In addition eelgrass rhizome connections break or rot after 1 – 2 years. This leads to clusters of physiologically independent rhizome units of varying size among which the division of labour through resource translocation is no longer possible (Alpert 1999; Cain 1990).

What may be the consequences of such a growth pattern on the breeding system? Let us assume that each rhizome unit has an equal probability of producing flowering shoots (this seems to be the case in eelgrass), which means that flowering shoots of the same genetic individual can equally well occur in the centre and on the edge of a clone. The mating landscape encountered by pollen released from a flowering shoot with an edge position in the

clone would differ widely from the mating landscape of a flowering shoot with a central position within the clone. In the latter case, in order to successfully outbreed, pollen would have to cross many flowering shoots with receptive stigma of the same clone (geitonogamy) before it would reach a flowering shoot of a different neighbouring clone. On the other hand, a receptive stigma from a flowering shoot in the centre of a clone would have a very low chance of receiving pollen from the outcrossing neighbourhood because it would stand at the low density end of the pollen fallout region. It is even likely that pollen reaching the edge of its clone will encounter a neighbouring genotype, which is actually a close relative of the original clone (biparental inbreeding), because seeds (and pollen) most often disperse over very short distances. Rhizome units may produce flowering shoots over several years and in each year the geometry of neighbouring rhizome units will change in the course of the growing clone and/or disturbance events. The consequences of the changing geometry on sexual reproduction have become known as the intrusion of clonal patterns on plant breeding systems (Handel 1985). It will be one of the main issues investigated in this thesis (see chapters I and IV).



Clonal structures in eelgrass (*Zostera marina*). This is an example of how rhizome units may be organized in a 1m^2 patch of meadow from a bird's perspective. The sum of all green units belong to genet I, all the red units to genet II. Genet I is further separated into several clusters (A – D) of rhizome units through intruding growth of genotype II.

3 - MAPPING EELGRASS CLONES WITH MICROSATELLITE MARKERS

The concept discussed in the previous chapter is not new, yet for many clonal plant species only recently it became possible to actually access the level of the genetic individuals. Eelgrass and many other clonal plants show physiological fragmentation, which prohibits the use of rhizome connections to track entire clones. In these cases the genetic individual can only be assessed with genetic markers that have sufficient resolution to distinguish individuals (Suzuki et al. 1999). Microsatellite markers are perfectly suited for such purposes because they usually show very high levels of polymorphism. In this chapter I want to discuss the necessary steps to get from the leaf tip of an eelgrass shoot from 3 m water depth to its microsatellite profile. The resulting data set transforms a patch of dense eelgrass meadow into a pixel image of the clone structure.

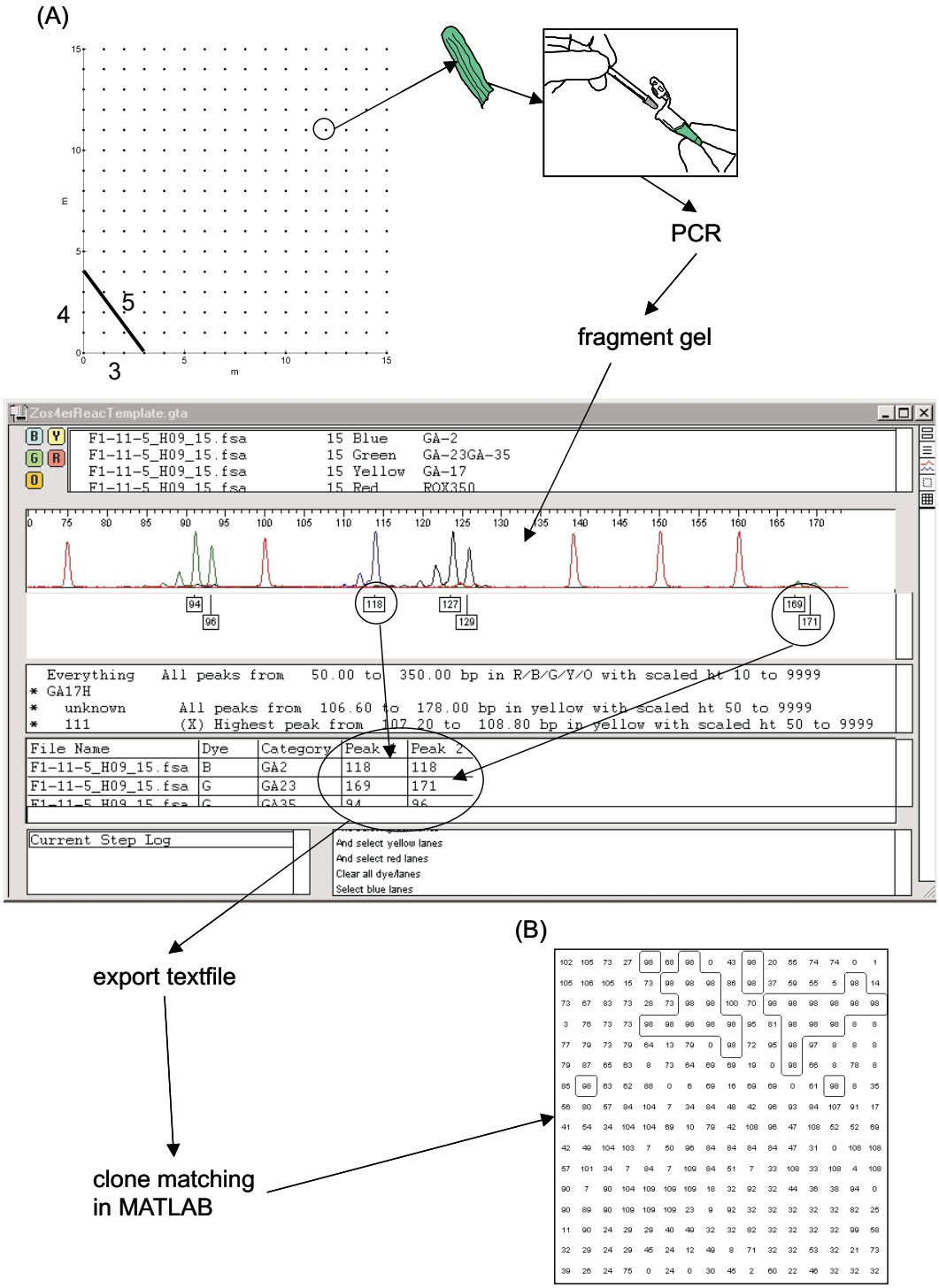
Microsatellites are single-sequence repeats on the DNA (short gene sequences), which have been found in every organism examined so far. They are made up of a single motif with a maximum length of six bases. These motifs are tandemly repeated which means that they are arranged head-to-tail without interruption by any other base or motif. Microsatellites have been shown to have mutation rates of up to 10^{-2} events per locus per replication, which is much higher than the average 10^{-9} commonly found for point mutations on other DNA stretches. This largely explains their high variability (Hancock 1999). The mutation pattern in microsatellites is assumed to follow the stepwise mutation model, which means that mutations only occur on the two ends of a microsatellite sequence with equal probabilities. This is a much-debated assumption that has not been verified so far but has great implications for the interpretation of microsatellite data (see Chapter II for more details). Another important point is that microsatellites show co-dominant inheritance and hence, homozygote individuals can be distinguished from heterozygote ones. The polymorphism of microsatellite markers can be genotyped with the polymerase chain reaction (PCR). In order to do so, one needs primers that bind to the conserved flanking regions of a microsatellite. Primers are short DNA sequences which have the property to bind to other specified DNA sequences. Because of the two strands in DNA there need to be a forward and a reverse primer, one of which is usually stained with a fluorescent colour molecule (primer ≈ marker) that can later be identified.

How can these primers now be used as probes for clones in an eelgrass meadow? The first step was to set up plots. In the case of this work, these plots where 15-m x 15-m squares located within closed eelgrass meadow in c. 3 m water depth. The plots were then marked with flexible posts in 1-m intervals. From a birds perspective this set-up looks like a

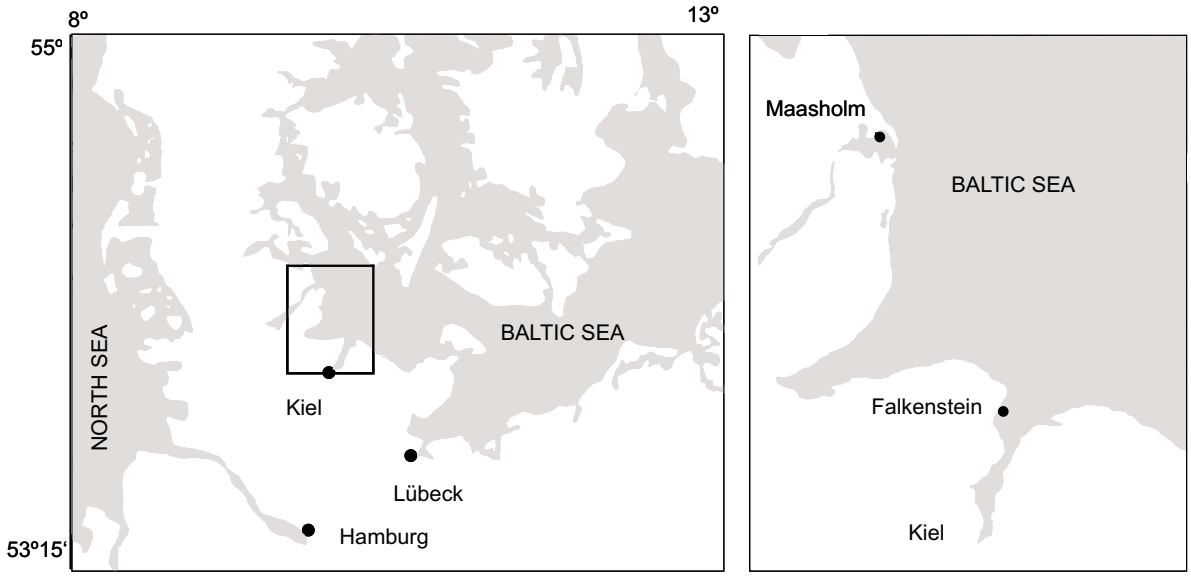
blank template of 256 pixels. Leaf tips of eelgrass shoots located closest to these grid points were collected and then dried. This first step resulted in a bag of tubes with dried leaf tips and each tube had the co-ordinate of its source location written on it. The leaf samples were then processed through several purification steps to get a solution with total genomic DNA from each sample. To amplify the microsatellite profile of each sample, DNA extract together with a cocktail of several substances (polymerase, bovine serum albumin etc.) including the microsatellite primers is processed in a polymerase chain reaction (PCR). The PCR reaction is basically kept running with a cyclic warming and cooling program during which the primers bind to their specific sequences and amplify them. The PCR products are solutions with a high concentration of amplified gene sequences of different length (different alleles) (Griffiths et al. 1993). Different sized sequences have different running speeds through an electronic field. This property is used to detect the different alleles in each sample. In this work, PCR products were run on an optical automated sequencer machine. A few µl of PCR product are applied to the beginning of a thin polyacrylamid – gel (each line a different sample) and fragments are separated through electrophoresis in buffer solution with high voltage (c. 3000 V). At the end of the gel the fragments pass a laser (optical sequencer) with smaller fragments passing earlier than larger fragments. The laser detects the fluorescent dye from the primers and transforms these signals into a virtual gel image on the computer screen. Finally the bands on the gel image can be translated into peaks (alleles) at different locations (size of allele) and height (different intensity of amplification). An internal size standard facilitates the determination of the exact size of each allele.

These peaks are then scored according to their size individually for each sample. The scores are written into a table, which can be exported as a plain text file for use in other applications. Samples processed through all these previous steps have yet received its microsatellite profile with two either identical (homozygote) or different (heterozygote) alleles for each analysed primer (up to 9 primers in this work). In a last step, all samples from one plot with identical microsatellite profile have to be matched because (assuming high enough resolution) these belong to the same genetic individual. For three clones and 10 samples this can easily be done by hand. With more than 1000 samples (256 from each plot, 4 plots) and several hundred clones it is better to have a computer program to perform this task. The program MATLAB provides a high-level language platform ideally suited to perform such tasks. It has many build in procedures (unlike C) and at the same time allows for maximum flexibility and speed (unlike JAVA). The codes for clone matching together with a sample start-up program are presented in appendix1 as reference (the program can be downloaded from http://www.mpil-ploen.mpg.de/english/physeco/staff/hammerli.htm). All the samples with iden-

tical microsatellite profile (identical genotypes) were assigned a unique number. Finally these sample numbers were plotted to the co-ordinates they were collected from, which transforms the previously blank template into a map of eelgrass clones, with a resolution of 1-m and a picture size of 256 pixels. And these maps (4 in total) build the foundation for the studies presented in chapters I – V. They either identified clone margins for the selection of replicated transplants both for laboratory and field experiments or their clone structure served as template for the measurements of reproductive output at the level of the genetic individuals. Apart from clone identification, depending on the number and variability of the used markers, microsatellites can also include information on recent mating events, on the level of inbreeding and on the level of genetic differentiation both within and between populations.



Schematic illustration of the main steps necessary to get from a patch of eelgrass meadow (A) to a genetic map of its clone structure (B). These steps are: establishing plots, collecting leaf samples, extracting DNA, amplify microsatellites (PCR), separate microsatellite alleles (fragment gel), score alleles, export allele data to MATLAB, and match clones. During the summer month the visibility in the Baltic Sea can be very low (< 1m). Despite this low visibility it is possible to set up exact rectangles with the use of Pythagorean triples. When starting from one corner with two measuring tapes, with the first marked at 3m, the second at 4m then the hypotenuse has to be 5m (hyp = $(3^2+4^2)^{1/2}$) (see grid in (A)).



Location of the sampling plots in Falkenstein and Maasholm on the Baltic Coast.

4 - THESIS OUTLINE

This thesis is organized in five chapters, each investigating a different aspect of the effects of fine scale clonal structure on population dynamics. The technical details of each study can be found in the specific chapters. Each chapter is organized in summary, introduction, methods, results and discussion. This outline is a very general introduction to the concepts of the research questions, explaining for each study why it was performed, what was the question and how it was tested.

The different aspects investigated were:

(I) Effect of pollen source and vegetative neighbourhood on inflorescence sex-ratio and seed production

(II) The effects of inbreeding depression on the size distribution of genets

(III) Local adaptation at the level of individual eelgrass genets

(IV) Contribution of three different levels of clonal organization on kinship structure

(V) Dynamics of flowering output and growth at the level of individual eelgrass clones

(I) In the first chapter I discuss an experiment for which I transplanted replicated genets into tanks under laboratory conditions. The motivation was to test whether flowering rhizome fragments could recognize the genetic quality of their neighbourhood. The reason why I

expected some recognition mechanism to be present in eelgrass is briefly explained in the following paragraph, which connects to the discussion on clonal organization in chapter 2 of this introduction.

In self-compatible hermaphroditic eelgrass and other clonal plants, pollen from a flowering shoot in the middle of a large clone has to travel a long distance to reach the next flowering shoot of a foreign clone to outcross with. In addition, pollen landing on the stigma of such a central flowering shoot will most often be self-pollen from neighbouring flowering shoots of the same clone. It may be advantageous for a flowering shoot to adjust flowering output (flexible mating) such that if available, outcross pollen can be favoured over the large amounts of self-pollen. Mate recognition in self-compatible plants known as cryptic self-incompatibility could be such a mechanism, but also recognition other than pollen (e.g. allelopathy) are possible. In a laboratory experiment I tested for mate recognition across several replicated genets of *Zostera marina*, focusing on the evolutionary context rather than the specific physiological mechanism underlying mate recognition.

(II) In the second chapter the aim was to investigate the effects of inbreeding depression, measured by individual heterozygosity, on the size distribution of clones. The starting point for this study was the observation that many clonal plants have a very skewed size distribution of genets. This is also true for *Zostera marina*. Until now it was suggested that these patterns were shaped primarily by stochastic processes (Suzuki et al. 1999). At the same time however, it was predicted that genet size correlated with fitness (Gardner & Mangel 1999). If true, then a deterministic process, namely inbreeding depression may be responsible for the skewed size distribution of genets found in most clonal plants. Furthermore, the outcome of this process should be visible in a correlation between individual heterozygosity (as a proxy for inbreeding depression) and clone size (as a proxy for fitness), which is what I tested in this study.

(III) In the third chapter I discuss an experiment aimed to test for local adaptation in eelgrass genets. I used a standard reciprocal transplantation design of replicated genets between the two populations Falkenstein and Maasholm. The presence of local adaptation in plants is the rule rather than the exception, because plants are sessile and their pollen and seeds usually disperse over short distances. What triggered this experiment was that local adaptation had not been tested in any seagrass species so far and because recently the discussion on how reliable genetic marker actually measure phenotypic performance has been fuelled. Marker diversity is commonly used to decide which populations are most suitable as a source for translocation and restoration (Haig 1998; Knapp & Rice 1998; Templeton 1986). The assump-

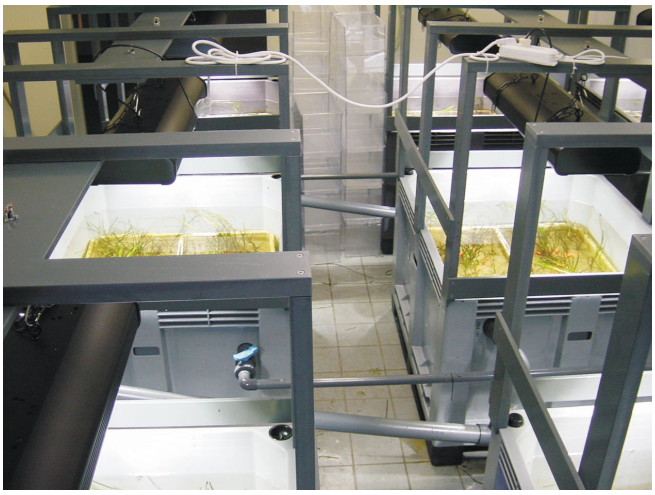
tion is that those populations possessing the greatest level of genetic variation are those with the greatest evolutionary potential in terms of establishing a new population (Moritz et al. 1995; Vrijenhoek 1994). However, locally adapted plants can suffer outbreeding depression if transplanted to a new environment to which they are not adapted (McKay et al. 2001). In this case, management decisions based only on marker diversity would be misleading. Hence, the motivation for this experiment was to support the analysis of within and between population marker diversity by measuring genetic variation in quantitative characters in a test for local adaptation.

(IV) In the fourth chapter I looked at spatial aspects of the neighbourhood structure in the sampled meadows. The method used in this study was spatial autocorrelation. Because clonal plants are spatially organized on three different levels (see chapter 2 of introduction) spatial autocorrelation has to be modified depending on the conclusions we want to draw. For example, if we want to test whether limited dispersal of seed and pollen leads to kinship structure ('family gathering') we have to exclude multiple samples with identical genotype from the analysis, because this would inflate the signal of spatial autocorrelation (identical genotypes are related to 100%). For this study I used MATLAB codes to prepare the genotypic data such that it represented either single rhizome units (ramets), clone fragments (spatially independent aggregations of ramets) and whole clones. I then performed spatial autocorrelation on these data sets to measure the contribution of the neighbourhood of each organizational level on kinship structure.

(V) Finally in chapter five I discuss a study of which I hope it will continue for several more years. In clonal plant research there is a great divergence between theoretical models and what is actually measured in the field (Eriksson 1993). Theoretical work on reproductive effort (Armstrong 1982; Gardner & Mangel 1999), clonal integration (Oborny et al. 2000), fitness components (Winkler & Fischer 1999) and senescence (Gardner Shea & Mangel 1997) has usually addressed the level of the genetic individual (the clone) while in contrast most empirical work has either investigated shoot demography (e.g. Donohue et al. 2000; Ishii & Takeda 1997; Nantel & Gagnon 1999) or has described genet composition (e.g. Ellstrand & Roose 1987; Kreher et al. 2000; Persson & Gustavsson 2001; Stehlik & Holderegger 2000). The main reason for this divergence were the difficulties to identify genetic individuals of clonal plants on a scale large enough to be relevant for population dynamics. The interface between models and field studies would be a true genet demography, the direct measurement of recruitment, mortality, growth and reproductive effort at the level of single clones. With the advent of high-resolution markers these constraints are now overcome. I resampled both per-

manent plots from the population of Falkenstein after one year in 2001, again for all the parameters measured in 2000. The clone maps together with reproductive output and density at the level of the genetic individuals from these two years were used to get first estimates on the dynamics of genets within the closed meadow.


Chapter - I



Sea water tanks for the pollination treatments in room A112 at the Institute for Marine Sciences in Kiel

Applying pollen to target flowering shoots

Flexible mating: experimentally induced sex-ratio shift in a marine clonal plant

Summary

Many hermaphroditic plants have evolved mechanisms to reduce interference between the sex functions and to optimise reproductive output. In addition to physical mechanisms such as the spatial (herkogamy) and temporal (dichogamy) separation of male and female functions, plasticity in sex expression by means of mate recognition (flexible mating) could be important in plants with variable access to foreign pollen. We experimentally tested for the effects of pollen source and vegetative neighbourhood on inflorescence sex-ratio and seed production in the self-compatible clonal marine angiosperm *Zostera marina* L., for which access to foreign pollen for flowering shoots is expected to depend on the position (central – peripheral) within the clone. In a laboratory experiment, we exposed flowering shoots to self and outcross pollen and to a neighbourhood of own and a mix of foreign vegetative shoots. Flowering shoots that had been exposed to outcross pollen showed (1) a significantly lower female/male ratio at peak flowering, evidence for mate recognition, and (2) a significantly higher seed set by the end of the season. Both effects were independent of the genetic composition of their vegetative neighbourhood. The results suggest that *Z. marina* maintains a cryptic self incompatibility system not previously described for angiosperms with sub-aqueous pollination. In *Z. marina*, and possibly other self-compatible clonal plant species, mate recognition could be a mechanism to increase the immediate outcrossing probability by delayed selfing for flowering shoots with central positions within their clone.

Introduction

In hermaphroditic organisms three main solutions are known to the problem of interference between the sex functions. Female and male organs can either be separated temporally or spatially, or female organs can discriminate between own and foreign gametes in order to control the degree of inbreeding. In hermaphroditic plants these solutions are known as dichogamy (temporal separation), herkogamy (spatial separation), and self incompatibility (Charlesworth, 1988; Bertin & Newman, 1993; Jarne & Charlesworth, 1993; Barrett, 1998). In self-compatible plants, dichogamy and herkogamy have been shown to reduce levels of inbreeding but also reduction in physical interference between sex functions can play a role (Fetscher, 2001). Given the modular growth form of many plants, the separation of female and male sexual organs is often not sufficient to eliminate selfing between flowers (geitonogamy) (Lloyd & Webb, 1986). In these cases different fertilisation abilities between self and cross pollen could play a role in controlling selfing rates (Levin, 1975; Schemske & Lande, 1985; Cruzan, 1990; Travers & Holtsford, 2000). This requires mate recognition from the mother plant, expressed as cryptic self incompatibility. Cryptic self incompatibility has been investigated in several wild plant species either through marker gene data (Bateman, 1956; Bowman, 1987; Casper et al., 1988; Johnston, 1993; Eckert & Barrett, 1994; Jones, 1994; Baker & Shore, 1995), pollen tube observations (Casper, 1985; Waser et al., 1987; Fenster & Sork, 1988; Hessing, 1989; Aizen et al., 1990) or a combination of both (Weller & Ornduff, 1977; Glover & Barrett, 1986; Cruzan, 1989; Snow & Spira, 1991; Montalvo, 1992; Cruzan & Barrett, 1993; Rigney et al., 1993). Most of these studies were directed towards the identification of the mechanism underlying differential pollination success. Surprisingly little is known about the context and the conditions in which such mechanisms could evolve (but see Eckert & Allen, 1997).

It has been suggested that such recognition mechanisms in self compatible plants have evolved to provide plasticity in sex expression (mating flexibility) under different ecological conditions (Barrett, 1998). One ecological condition that could promote the evolution of mating flexibility is the growth form of clonal plants. In this large group of species access to foreign pollen is expected to decrease sharply with increasing distance to the clone edge and with this the level of between flower selfing (geitonogamy) increases (Handel, 1985; Eckert, 2000). Given that flowers are abundant, between flower selfing will be strongest in single species stands where genets can cover extensive areas, as in many aquatic flowering plants (Barrett et al., 1993). Also wind or water pollinated plants should be prime candidates for studying local variation in pollen access because the shape of pollen fallout curves is only

determined by the physical properties of the transporting medium and the pollen morphology.

A genetic individual of a clonal plant can be described as a local aggregation of potentially physiologically independent units (ramets, Harper, 1977). It has been shown that clonal plants use intact root connections (clonal integration) to relocate resources in response to environmental conditions (eg. optimal foraging, de Kroon, 1995; Evans & Cain, 1995; Stoll et al., 1998), within the limits of the costs associated with plasticity (Kleunen et al., 2000). However, in many clonal plant species these root connections rot away or break over time, leaving physiologically independent units behind (Pitelka & Ashmun, 1985; Groenendael & Kroon, 1990). The disposition for physiological fragmentation is adding to the difficulties in sexual reproduction within larger clones. While interconnected ramets can in principal show a spatial division of labour to optimise reproductive output (e.g. only peripheral ramets flower) (Charpentier & Stuefer, 1999), single physiologically independent units within a cluster of fragments have unequal opportunities for outcrossing depending on their position (central or peripheral) within the clone (Handel, 1985).

The aim of the present study was to investigate mate recognition in a self compatible plant species in an approach that identifies the conditions in which such mechanisms have evolved. We were interested in a possible evolutionary context rather than the specific physiological mechanism underlying mate recognition. Our study system was eelgrass (*Zostera marina* L.) a marine angiosperm which forms dense meadows with clones that can cover several 10 m^2 of area (Reusch et al., 1999a). Pollination is sub-aqueous with short dispersal distances (1 - 3 m) (Ruckelshaus, 1996) and fitness costs through geitonogamous selfing were found to be substantial (Reusch, 2001).

In the present study we experimentally tested for the effects of pollen source and genetic composition of the vegetative neighbourhood on inflorescence sex-ratio and seed production of target flowering shoots in *Zostera marina*. The prediction was that in the presence of a flexible mating system, sex expression (female/male spikes) of single independent rhizome fragments would be dependent on the position within their clone and on the pollen arriving at reproductive shoots. Because clonal plants are expected to perceive their neighbourhood not only through pollen but also through differences in the competitive regime (de Kroon, 1993; Hubersannwald et al., 1997), we included genetic composition of the vegetative neighbourhood as treatment factor. We exposed flowering shoots of the marine clonal plant *Zostera marina* to two extreme positions within the genet under laboratory conditions. Target shoots were either exposed to self (central position) or a mix of cross (peripheral position) pollen. As an orthogonal treatment target shoots were surrounded by units of the same clone (simulating central position) or surrounded by units of a mix of different clones (simulating

peripheral position). The effects were tested on the sex-ratio to the level of single inflorescences and seed production. We expected a shift in the ratio of inflorescence bearing female and male flowers as short term response, and a lower seed output for flowering shoots with central positions as longer term response.

Materials and methods

The study species

Eelgrass (*Zostera marina* L.) is a submerged flowering plant that is found in temperate seas of the northern hemisphere (Den Hartog, 1970). In the non-tidal Baltic sea *Z. marina* is perennial and forms meadows with clones of several 10 m^2. In the south-western Baltic (Åland) some clones were estimated to be > 1000 m^2 (Reusch et al., 1999a). The plant forms distinct flowering shoots that can easily be distinguished from vegetative shoots in an early stage of development. Flowering shoots are branched with up to 14 inflorescences, each bearing several female and male flowers (monoecious) and flowering phenology is protogynous at the level of single inflorescences (spadix) (De Cock, 1980). The genetic individual is a functional hermaphrodite. Hand pollination has shown that *Z. marina* is self compatible (Ruckelshaus, 1995). The filamentous morphology of the pollen has been interpreted as an adaptive trait for sub-aqueous pollination (Cox, 1983; Ackerman, 1997).

Mapping of genets

In order to obtain plants with known genetic identity, high resolution clonal mapping was conducted in the field. During the month May and June 2000 two 15 - m x 15 - m permanent plots were set up in shallow water eelgrass beds (2 m – 3.5 m depth) in Falkenstein (Schleswig Holstein, Germany) along the Baltic coast. Both plots were sampled on a 1-m regular grid (256 points). Leaf material of the plants closest to the grid points was collected in an non destructive way and preserved in silica-gel for DNA amplification. The four most polymorphic microsatellite markers for *Zostera marina* (GenBank accession no. AJ249307, AJ249305, AJ009900, AJ009898) were used in a combined PCR-amplification with 26 cycles. PCR conditions and gel electrophoresis on an ABI 377 genetic analyser followed standard protocols (Reusch et al., 1999b).

Microsatellite alleles were scored with the software package Genotyper (Biosystems, 1998) and all typed samples of one distinct genotype in each plot were treated as one clone. This produced 256 pixel images of the genet composition in the two plots. Because the sampled plots were close replicates in terms of clonal diversity (number of distinct multilocus genotypes/number of samples) and size distribution of genets (Hämmerli & Reusch unpubl. data) they seemed to be representative for the population structure in this area.

Experimental set-up

In a tank experiment we tested for a shift in sex expression (inflorescence sex-ratio) and differences in seed production as response to pollen source and genetic composition of the vegetative neighbourhood in flowering shoots of *Z. marina*. In the literature the term 'ramet' is usually reserved for a potentially independent module in a clonal plant (Harper, 1977). In this work we addressed the actually independent rhizome fragment of a genet found in the field which we call 'unit' to avoid confusion.

The experiment was set up as a randomised block design with 9 blocks containing 4 plots each (Fig. I.1). The blocks were tanks (1120 mm x 1000 mm x 765 mm) filled with ambient Baltic Sea water that was continuously exchanged every 45 minutes. The tanks were subdivided into four sub-compartments each filled with sediment to a height of 13 cm. Each tank was equipped with a light panel (2 x 150 W, HQI), 50 cm above the water surface. Typical light levels were 20 W m^{-2} s^{-1} (water surface) and 5 W m^{-2} s^{-1} (sediment surface).

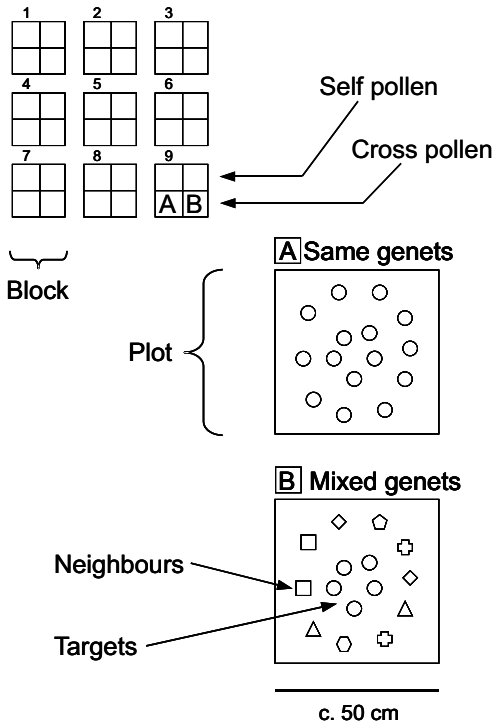


Fig. I.1: Experimental set-up for the tank experiment with *Zostera marina*. Each tank (block) contained a different target genotype (1 - 9) and was divided into 4 sub-compartments (plots). Each plot contained 5 target flowering shoots in the centre surrounded by a neighbourhood of 10 vegetative shoots of either the same genet as the target shoots, or a mix of different genets. Self pollen or a mix of outcross pollen was added to the target shoots as second orthogonal treatment factor.

Based on the genotype maps 9 genets were randomly selected out of all genets covering an area of ≥ 3 m^2 (n = 23). Between 9 – 12 Mai 2001, 20 units

with flowering shoots from each of the 9 selected genets were excavated (targets). Special care was taken not to break the root connections other than within the rotten parts. This way, we harvested the effectively independent units rather than the potentially independent units or ramets. All units had only one flowering shoot, 30 % of which had 1-2 additional vegetative shoots. The targets were individually marked and groups of 5 were planted in the centre of the plots, one genotype per tank (Fig.I.1). At the same time each group of 5 target flowering shoots was surrounded by vegetative shoots of either the same genet as the target shoots or a mixture of 6 different genets (neighbourhood) (Fig. I.1). For the production of pollen, additional flowering shoots from each of the 9 target genets and from the genet mix (6 genets) were harvested and loosely planted in separate aquaria for flower ripening.

After the onset of flowering the shoots of the target ramets were covered by cylinders of nylon mesh (100 μm) from the sediment to the water surface with a volume of 15.5 L of water surrounding the 5 targets. Following De Cock, 1980 and Pettit, 1984, the chosen mesh size should limit pollen exchange between the treatments within each tank and at the same time allow for water exchange. This could be confirmed with microscope images of collected pollen (> 10 x 400 μm).

From the aquaria of pollen producing flowering shoots, spikes that released pollen were harvested and placed in tubes to produce pollen suspensions. Because *Zostera* pollen has been reported to be short lived (c. 48 h) (De Cock, 1980) care was take to use only spikes with thecae just releasing pollen which was visible as milky exudate close to the anthers. This suspension was then pipetted into the nylon mesh tubes. Pollen suspensions were standardized by the number of spikes harvested per pollen treatment but could vary between days depending on the amount of pollen releasing spikes available (6 – 10 spikes / 50 ml). Typical pollen concentrations were 322 ± 23.25 SE (n = 20) in 2 ml of suspension. From this we roughly estimated the background level of own pollen in the water column released by the target shoots to be 90 – 150 times higher at the point of measurement and 3.5 – 6 times higher for the rest of the treatment period as compared to the pollen added by the treatment, because of the higher number of spikes releasing pollen within the treatment units as compared to number of spikes in the suspension.

The pollen treatment lasted 6 - 24 June. During this period, the mesh cylinders were removed and washed twice > 48 h after the last pollen treatment to avoid life foreign pollen dispersing to other plots within the tanks. On 10 June flowering shoots were measured for number of spikes. The spikes were scored as i) unripe (no pistil visible), ii) female (> 1 pistil visible) and iii) male (>1 theca releasing pollen) flowering stages. Protogyny within inflorescence was not always 100 % which is in line with findings from (Ruckelshaus, 1995). Thus

the scoring method used might have slightly underestimated the overall number of spikes with receptive pistils. After the second week of the pollen treatment only few pollen releasing spikes were left with approximately 90 % in past male flowering stages. Three weeks after the onset of flowering seed set was visible as a swelling of the spadices. Seeds were counted approximately one week before the first spadices opened to release seeds to ensure that seeds had not fallen out before. A random subset of the seeds from each plot was dried at 80 °C and weighed on an electronic analytical balance (Sartorius analytic A120S) to the closest 1 x 10^{-4} g. Target shoots were excavated after the experiment and measured for rhizome length and number of vegetative shoots in addition to the flowering shoots.

Statistical analysis

Plants from each of the 9 genotypes were randomised over the plots within a block. For the measured variables at the time of planting, rhizome length, shoot height and number of spikes, all F were < 2.5 and all P were >> 0.05.

The ANOVA model for flowering (fraction of 'male' spikes/ fraction of 'female' spikes) and seed production (mean number of seeds/spike) contained two treatment factors (genetic neighbourhood; pollen) and one random factor (genotype within block). This yielded F-ratios with 24 degrees of freedom (df). All analysis were done with the software package GENSTAT (Payne, 1997). Note that the correlation's were calculated with means over 5 target shoots and thus their F-ratios have 34 df.

Results

Flowering

At the time of planting the target units were bearing 26.5 % of the total produced spikes. One week after the onset of flowering, 76.3 % of the total inflorescence were visible with proportions of 22.1 % female, 47.8 % male and 30.0 % unripe spike stages. After two weeks, the proportion of spikes in these three stages dropped to 5 -10 %, thus the main flowering output took place within two weeks. The pollen treatment had a significant effect on the ratio of spikes with female flowers to spikes with male flowers ($F_{1,24}$ = 9.25; P = 0.006). The ratio was lower for target shoots that had been exposed to a mix of cross pollen (Fig. I.2a).

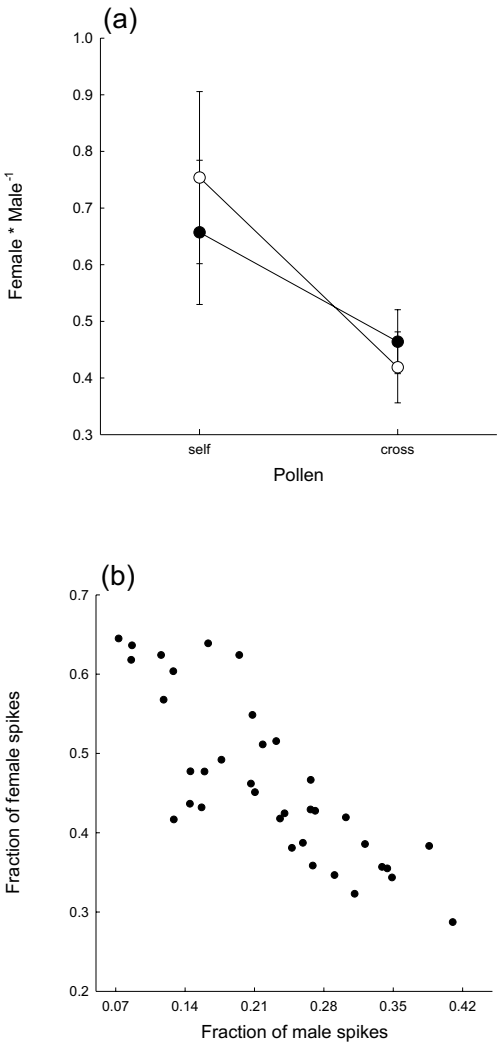


Fig. I.2: Mean ratio of female / male spikes for the treatments of own and a mix of cross pollen and vegetative neighbourhood of the same genet (closed circles) and a mix of different genets (open circles) (a). Correlation between the fraction of spikes in female stages and the fraction of spikes in male stages (b).

Averaged over the 5 target shoots per plot, the proportion of spikes with females flowers was significantly negatively correlated with the proportion of spikes with male flowers (R = - 0.65; $F_{1,34}$ = 66.54; P < 0.001) (Fig. I.2b), showing strong spike level dichogamy. The genetic composition of the vegetative neighbourhood had no significant effect on the spike sex-ratio ($F_{1,24}$ = 0.09; P = 0.770) (Fig. I.2a). There was no significant difference in the total number of spikes for both treatments (pollen: $F_{1,24}$ = 1.07; P = 0.310; neighbourhood: $F_{1,24}$ = 0.38; P = 0.544). None of the interactions were significant.

Seed production

Mean number of seeds per spike was significantly lower for flowering shoots that had been treated with self pollen ($F_{1,24}$ = 5.42; P = 0.029). In contrast there were no significant differences between neighbourhood treatments ($F_{1,24}$ = 0.55; P = 0.464) (Fig. I.3). There was no significant correlation between initial rhizome length and seed production (R = 0; $F_{1,34}$ = 0.00; P = 0.994). Initial shoot height and mean number of spikes per shoots of the targets were weakly but not significantly negatively correlated with seed production (R = - 0.070, - 0.074; $F_{1,34}$ = 3.65, 3,81; P = 0.065, 0.059). There was no significant difference in the dry weight of single seeds for both treatments (pollen: $F_{1,24}$ = 0.8; P = 0.381: neighbourhood: $F_{1,24}$ = 2.18; P = 0.152). As for the variables associated with flowering, no interactions were significant.

Rhizome growth and vegetative shoots

As mentioned earlier 30 % of the excavated target units had 1 – 2 vegetative shoots in addition to the flowering shoot. At the time of planting, mean overall rhizome length for targets with vegetative shoots was 6.74 cm ± 0.270 SE (n = 127) and 6.52 cm ± 0.405 SE (n = 52) for targets without vegetative shoots. At the time of harvest rhizomes grew to 6.76 cm ± 0.280 SE (n = 131) and 13.21 ± 0.653 SE (n = 48), respectively. Out of the 180 targets planted, 7 had lost the vegetative shoots during the experiment, while only 2 had produced new vegetative shoots.

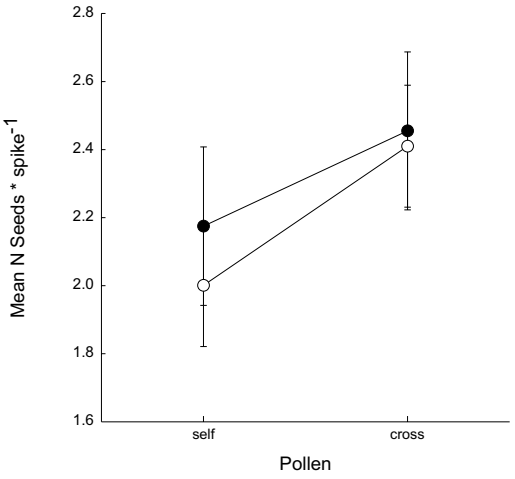


Fig. I.3: Mean number of seeds per spike for the treatments of own and a mix of cross pollen and vegetative neighbourhood of the same genet (closed circles) and a mix of different genets (open circles).

Discussion

Effects of pollen treatment

Target flowering shoots exposed to a mix of foreign pollen had a 37 % lower female / male – spike ratio at peak flowering and produced 16 % more seeds per spike at the end of the season (Figs. I.2a, I.3). Because the experiment was controlled for the amount of pollen, the difference in female / male – spike ratio must have been a response to the pollen source rather than the quantity. Because flowering is sequential at the level of single spikes, observed male spikes were past the female stage. This is also strongly reflected in the negative correlation between the fraction of female to the fraction of male spikes (Fig. I.2b). The pollen treatment had no significant effect on the total number of spikes. Thus the most likely explanation for

the observed shift in the instantaneous floral sex-ratio in response to the pollination treatment is that it resulted from changes in the functional life-span of female flowers. Target flowering shoots that had been exposed to a mix of cross pollen showed more spikes already in a male stage at the time of measurement, while targets that had been exposed to self pollen showed a higher proportion of females that remained receptive. It has been suggested that many species considered to be self-compatible on the grounds of seed set from cross- versus self-pollination, may posses self-recognition mechanisms eg. cryptic self incompatibility or other types of pollen-pistil interactions (Barrett, 1998). The observed shift in the spike sex-ratio strongly suggests such a mechanism to be present in *Z. marina*. In addition we must assume that the underlying mechanism is very efficient, as a ratio of approximately 1 outcross : 100 self pollen was sufficient to produce the observed effect (see methods). Inbreeding depression could in principal have contributed to the observed shift in the spike sex-ratio. The response, however, was short term, measured five days after the first, and three days after the second pollen treatment. Even if a fraction of the ovules had already been fertilised before the measurement, it seems very unlikely that the observed ratio shift would have been a response partly triggered by inbreeding depression.

Inbreeding depression vs. cryptic self-incompatibility

The significantly lower levels of seed set with own pollen is showing reduced relative fecundity upon selfing in this species which is in line with findings from (Ruckelshaus, 1995). However, mate recognition through a post-pollination mechanism could in principal have similar effects on seed set as pure inbreeding (Nettancourt, 1977; Charlesworth, 1988; Hiscock et al., 1996). Thus, we cannot decide whether lower seed set is only caused by inbreeding depression in the sense of homozygosity of deleterious recessive alleles. The observed reduction in seed set in *Z. marina* could have been the result of a combination of inbreeding depression and cryptic self-incompatibility. In a random sample of shoots collected from the source population the levels of seed set in the field were in the same range as for target shoots exposed to a mix of cross pollen in the laboratory (2.63 ± 0.24 SE (n = 10) field, 2.43 ± 0.14 SE (n = 18) laboratory). Keeping in mind that the local concentration of endogenous self pollen is expected to be much higher than the cross pollen concentration, similar seed set in the field may indicate that pollen discrimination is also active in the wild population. It is clear however, that conditions in the field are different from the ones in the laboratory and therefore such comparison have to be treated carefully. Although technically difficult to obtain (isolate single female flowers from self pollen in the water column) it would be interesting to

have estimates on seed set after pure cross pollination as comparison to the results of this study and data from the field.

Genetic composition of vegetative neighbourhood

Only 30 % of the flowering shoots that were excavated as intact physiologically independent unit of a genet had additional vegetative shoots. These 30 % doubled the rhizome length during the experiment, whereas the rhizomes of targets with no vegetative shoos did not grow. Rhizome growth of flowering targets with vegetative shoots closely resembled measurements from units with only vegetative shoots transplanted in the field. In the latter group rhizome length changed from 5.99 cm ± 0.274 SE (n = 99) to 16.74 cm ± 0.727 (n = 76) by the end of the season (Hämmerli & Reusch, unpublished data). In addition rhizome length of single units at the beginning of the experiment did not predict seed output. These findings were surprising and counter to our expectation and they seem to indicate that the development of flowering shoots in *Z. marina* is largely independent from their rhizome. This may in part explain why there was no significant effect of the vegetative neighbourhood on the measured variables because in the absence of allocation between rhizome and flowering shoots, differences in competitive interactions could hardly be expressed as differences in the development of inflorescence and seeds.

Clonal growth geometry and plasticity in sex expression

Single *Z. marina* clones can cover large areas with presumably high endogenous concentration of self pollen. In the sample population used in this work we found an average of 20 flowering shoots m^{-2} ± 1.3 SE (n = 256), with each bearing about 5 male and 2 female spikes per shoot at peak flowering. This adds up to an estimated 100 pollen releasing spikes and 40 spikes with receptive stigmas flowering simultaneously in one m^{-2} of meadow. This means that dichogamy is only achieved at the level of single spikes. At the level of whole shoots or even clones *Z. marina* is a simultaneous hermaphrodite and it seems unlikely that dichogamy can have a significant influence on the degree of selfing. Pollen recognition in this species may provide mating flexibility for flowering shoots which stand at the low density end of the pollen fallout region (eg. central position) from a neighbouring genet because they could increase the probability for outcrossing by delayed selfing (sensu Lloyd & Webb, 1986; Lloyd & Schoen, 1992). Eckert & Allen, (1997) investigated cryptic self-incompatibility in *Decodon verticillatus* by measuring pollen tube growth. They hypothesised that such mechanisms

could be generally adaptive in species that exhibit strong inbreeding depression, experience great variation in the availability of outcross pollen and selection for maximum fecundity during any reproductive bout. *Z. marina* is known to suffer inbreeding depression and generally shows high outcrossing rates (Ruckelshaus, 1995; Reusch, 2000; Reusch, 2001; and this study). For any given reproductive unit the availability of outcross pollen is expected to change continuously both in time (water movement) (Ackerman, 1997) and space (as a function of geometry of the local neighbourhood through: fragmentation, clonal growth, disturbance) (Olesen & Sand-Jensen, 1994). The third requirement, selection for maximum fecundity is difficult to measure for any long lived perennial. However, because flowering shoots of *Z. marina* develop largely independent from their rhizome it seems likely that there is selection for maximum reproductive output at the level of single flowering shoots in each reproductive period. Resources invested into flowering shoots cannot be withdrawn and used for clonal growth or future reproduction.

Conclusion

Mate recognition is the prerequisite for a flexible mating system in self-compatible plants. Our results suggest that a mechanism for mate recognition through pollen is present in *Z. marina*, which would be the first detected for any angiosperm with sub-aqueous pollination. The proximate mechanism remains to be investigated. In the case of a self-incompatibility system we would expect it to be gametophytic (inhibition of growing pollen tube) rather than sporophytic (inhibition of pollen on the stigma surface) (Hiscock et al., 1996) because the filamentous pollen of *Z. marina* lack a pollen coat. In clonal plants where flowering shoots have no root connections between central and peripheral positions, isolation from potential outcrossing partners due to the increasing distance to the clone edge could be a selective force in the evolution of flexible mating systems.

Plasticity in sex expression or flexible mating may be common among self compatible clonal plants with large genets. The most promising candidates will probably be species with high levels of own pollen and at the same time high outcrossing rates. More data, in particular from field populations is needed to corroborate the requirements for an adaptive explanation of mate recognition. A more comprehensive evolutionary interpretation of mate recognition in long lived clonal plants will require data from other clonal species that exhibit cryptic or partial self incompatibility to assess whether the required conditions are generally met by a broad range of taxa. Further investigations are needed to increase our understanding of flex-

ible mating and its role among the diversity of breeding systems found in flowering plants.

Acknowledgements

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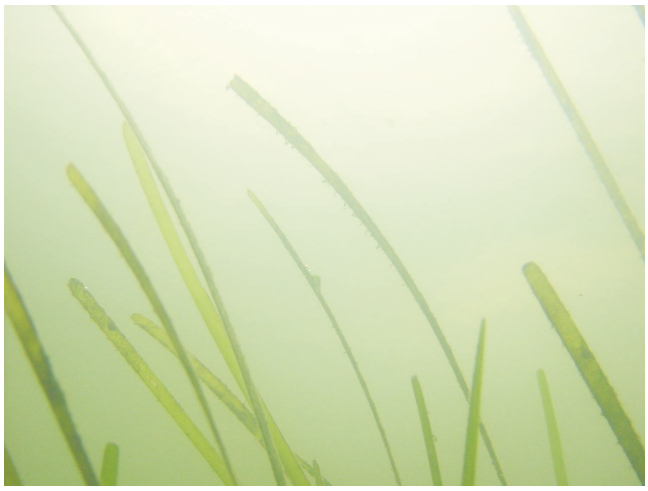
Chapter - II



Sun rays over Maasholm from an eelgrass perspective

Eelgrass flowering shoot soaring upwards above the canopy

Inbreeding depression influences genet size distribution in a marine angiosperm

Summary

In clonal plants genets are usually found to be of unequal size, yet surprisingly little is known about the evolutionary processes responsible for such a skewed size distribution. Here we test the prediction that the genet size distribution of the marine clonal plant *Zostera marina* (eelgrass) is influenced by inbreeding depression. We used 9 polymorphic microsatellite markers to access the fine scale clonal structure and to measure individual heterozygosity within 4 plots each corresponding to 256 m^2 sampled in 1-m intervals in two populations along the German Baltic Coast. The same plots were also sampled for flowering and vegetative shoots to obtain estimates for sexual reproductive output at the level of the genetic individual. We found substantial differences in genet size distribution between the two populations that may be explained by different disturbance frequency. In both populations, clone size was significantly positively correlated with the total number of flowering shoots, indicating that larger clones have a higher reproductive output. Individual heterozygosity was positively associated with clone size. The effect was much stronger in Falkenstein (low disturbance) than in Maasholm (high disturbance). The results indicate that in a low disturbance population the relatively outbred clones occupy a higher proportion of the available space, most likely because they outcompete relatively inbred neighbours. Interestingly, our data indicate that at the highest levels of individual heterozygosity, such fitness gains are no longer present, indicating an optimal level of outcrossing in a clonal marine plant.

Introduction

"What is a Dandelion ?" asked Janzen in 1977. Since then it has become evident that in clonal plants, the unit we observe in the field is usually not the genetic or 'evolutionary' individual (Janzen, 1977). Rather, clonal plants occupy space by reiterating modules that either remain physiologically connected, or more often break after some time, leaving clusters of fragments from the original individual behind (Groenendael & Kroon, 1990; Pitelka & Ashmun, 1985). The ability for extensive two dimensional expansion together with spatial fragmentation make clonal plants particularly interesting model species in the context of inbreeding and reproductive output (Eckert, 2000; Gardner & Mangel, 1999; Handel, 1985). At the same time, the potentially infinite lifespan of clones (= genets) makes life-history hard to track. And the tendency for clones to become fragmented makes genetic individuals hard to identify. This has brought up the need for molecular markers for studies on wild populations (Groenendael & Kroon, 1990; Klimes et al., 1997).

A wide range of molecular markers has been used in studies on the fine scale genet composition in clonal plants, e.g. allozymes (Godt & Hamrick, ; Hossaert-McKey et al., 1996; Ivey, Richards, 2001; Parks & Werth, 1993; Stehlik & Holderegger, 2000; Waycott, 1995), isozymes (Kudoh et al., 1999), RAPD (Auge et al., ; Gabrielsen & Brochmann, 1998; Kreher et al., 2000; Persson & Gustavsson,), AFLP (Pornon, Escaravage, 1999) and microsatellites (Procaccini & Mazzella, 1998; Van Der Velde et al., 2001 Reusch, 1998). Independent of the used markers however, many studies found that clones varied greatly in size with usually many small clones and a few larger clones in the population. Yet, surprisingly little is known about the potential evolutionary processes responsible for such skewed size distribution (Eriksson, 1993; Harada, Iwasa, 1996). Genet size distribution may be shaped by stochastic processes (Suzuki et al., 1999). However, an alternative, deterministic explanation for the observed patterns may be derived from predictions on the growth and reproductive output of clonal plants. Genet size has been found to be associated with an increased potential for sexual reproduction (Magda et al., 1993; Watson, 1984) and a reduction in genet mortality (Cook, 1979; Eriksson & Jerling, 1990). Based on these findings was the prediction that the size of a genet (e.g. the number of modules with identical genotype) correlates with fitness (Gardner & Mangel, 1999). If clone size correlates with fitness, inbreeding depression can be expected to influence the size distribution of clones because there will be selection for clones able to outcompete their relatively inbreed neighbours in terms of spatial expansion. As in other long-lived organisms, direct measurements of inbreeding depression for clonal plants with potentially infinite life-span is nearly impossible (Hutchings & Ferguson, 1992) because it

would require knowledge on the relatedness of individuals across several generations. To circumvent this difficulty, another approach that has proven successful for some animal (Coltman et al., 1998; Slate et al., 2000) and plant species (Bijlsma et al., 1994; Ouborg et al., 1999; Ouborg & Vantreuren, 1995; Willis, 1993), is to measure heterozygosity at genetic marker loci (Charlesworth & Charlesworth, 1987) under the assumption that any reduction in individual heterozygosity is indicative for selectively relevant genes as well (David, 1998; Hedrick, 1999; Hedrick, 2001; Parks & Werth, 1993; Roff, 1997; Sunnucks, 2000; Tsitrone et al., 2001). We thus predict that the size distribution of genets in a clonal plant has a deterministic component. We expect a positive correlation between genet size and individual heterozygosity. Such a relationship should be observable mainly at sites with moderate to low physical disturbance where dominant clones have enough time to grow large and outcompete other genotypes.

The aim of the present study was to test, 1) whether clone size in the marine angiosperm *Zostera marina* is a fitness correlated trait and 2) whether clone size correlates with individual heterozygosity. To this end the fine scale genetic structure was assessed with high resolution microsatellite markers in two eelgrass populations, assumed to differ in disturbance frequency. This gave estimates for the size-distribution of genets and individual heterozygosity. In addition the flowering intensity was measured as an estimate for reproductive output.

Materials and methods

Species, study area and populations

Eegrass (*Zostera marina* L.) is the dominant seagrass species of the northern temperate zone forming dense meadows through clonal growth in the non-tidal Baltic Sea. Its leaf canopy and root structure has significant importance as habitat for invertebrate and fish and for the stabilisation of the coastal sediment (Den Hartog, 1970). Flowering shoots are produced once a year and can be easily distinguished from vegetative shoots in an early stage of development. Flowering shoots start to grow in winter and early spring, while flowering and seed ripening takes place during the summer months (De Cock, 1980; Den Hartog, 1970). The plant is a functional hermaphrodite (monoecy) and hand pollination has shown that *Z. marina* is self compatible (Ruckelshaus, 1995). The filamentous morphology of the pollen has been inter-

preted as an adaptive trait for sub-aqueous pollination (Ackerman, 1997; Cox, 1983). Although seed production is abundant, in closed, well established meadows, seedling recruitment and establishment is rare (Olesen & Sand-Jensen, 1994a; Olesen & Sand-Jensen, 1994b; Robertson & Mann, 1984 & personal obs.) and thus open space is mainly colonised through branching and the horizontal growth of rhizome fragments.

Sampling took place in two sites along the German Baltic Coast separated by c. 80 km of coastline (Fig. II.1). Both sites are home to a natural population of *Z. marina*. The 'Falkenstein' population (plots F1 and F2) grows on the west side of the Kiel Förde, Schleswig Holstein (54°24' N, 10°12' E) in 3 - 3.5 m water depth. The 'Maasholm' population (plots M1 and M2) grows in an estuary at the mouth of the river Schlei, Schleswig Holstein (54°41' N, 10°00' E) in 1.5 - 2.5 m water depth. Both sites belong to the Kiel bight water body and therefore have similar salinity and temperature regimes (Reusch, 1998). The 'Maasholm' population grows in an embayment (Fig. II.1) and depending on the speed and direction of the wind the water level can vary up to 60 cm (Marahrens, 1995). The shallower waters of Maasholm make this population prone to disturbance by swan grazing and ice scour.

Mapping of genets with microsatellite markers

To assess the fine scale clonal structure, high resolution genet mapping was conducted in Falkenstein and Maasholm. During the month May and June 2000, two 15-m x 15-m permanent plots were set up in each population (F_1 and F_2, M_1 and M_2), using SCUBA (Fig. II.1). Each plot pair was located within c. 100 m of each other. All the plots were permanently marked with PVC posts in 1-m intervals at the base and the top line. With the aid of a measuring tape attached to these posts, each grid-line was marked in 1-m intervals with tagged flexible posts. The plots were sampled on a 1-m regular grid (256 points). Leaf material (3 - 5 cm) of the plants closest to the grid points was collected and preserved through drying in silica-gel for DNA amplification. If no plant was present within a radius of 10 cm around the grid point the position was counted as non vegetated. Total genomic DNA was extracted with the Qiagen plant DNA extraction kit (Qiagen, Hilden, Germany). The DNA-extract was processed in two combined PCR-amplifications with 24 and 28 cycles (see Table II.1), using 9 polymorphic microsatellite markers specifically designed for *Zostera marina*. PCR-reactions with fluorescently labeled primers followed standard protocols (Reusch et al., 2000). Alleles were scored on ABI 377 and ABI 3100 automated sequencers, using the software packages GeneScan 3.1 or 3.7 and Genotyper 2.0 or 3.7 (Biosystems, 1998; Biosystems, 2001). This produced 256 pixel images of the genet composition in the plots (Fig. II.1). A total of 947 sam-

ples was genotyped for all 9 loci. Very few samples were not successfully genotyped (F_1 = 9; F_2 = 0; M_1 = 4; M_2 = 7) and these were excluded from the analysis. The remaining empty pixels were grid points from which no leaf samples could be collected and thus corresponded to non-vegetated space at the time of sampling. The relatively low number of empty pixels (F_1 = 1; F_2 = 3; M_1 = 43; M_2 = 10) indicates that plots were positioned within a larger area of the closed meadow. The likelihood that ramets were erroneously assigned to the same genet because they exhibited the same nine-locus genotype by chance was very small (all P_{gen} << 0.001) (Parks, Werth, 1993) and therefore all typed samples of one distinct genotype in each plot were treated as a clone. No genotype occurred in more than one plot.

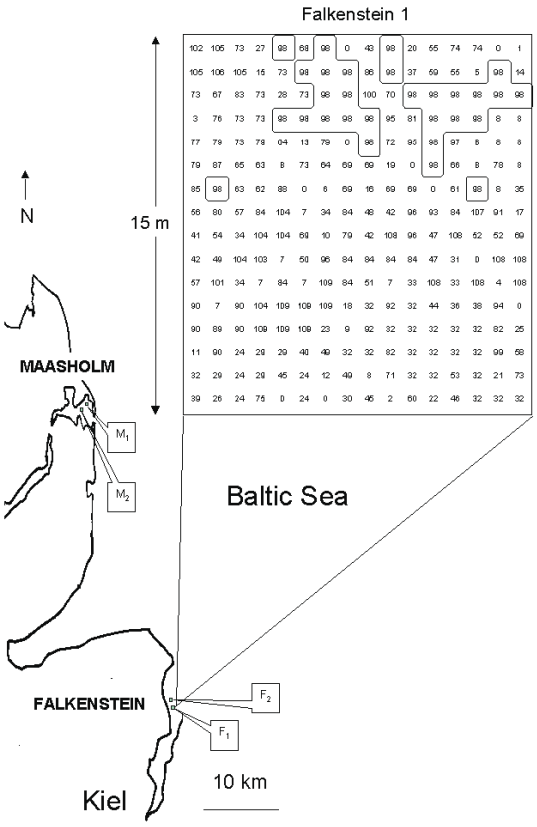


Fig. II.1: Two plot pairs (F_1-F_2 & M_1-M_2) in each of two populations (Falkenstein & Maasholm) along the German Baltic Coast with the genetic map of one of the plots from Falkenstein. Running numbers represent the distinct multilocus genotype found for leaf samples from each grid point that were typed for 9 polymorphic microsatellite loci. The plot is also showing nearest neighbour contours for genet number 98. According to our definition this genet represents one clone with an area of 29 m^2 (number of sampling points with identical genotype x 1 m^2).

The likelihood of erroneous clone assignment P_{gen} is based on the assumption of random allele recombination for each locus (Hardy-Weinberg equilibrium HWE) and no linkage between loci. These assumptions were tested with GENETIX (Belkhir, 2001). All loci were found to be in HWE (95% confidence interval: -0.00178 < 0.01539 < 0.03222, 1000 bootstraps) but in some pairings of the loci, linkage could be detected (overall 11 out of 144). In a key study on linkage disequilibrium in a house fly population Black & Krafsur (1985) detected significant linkage only in two loci comparisons with R_{ij} = 0.494 based on 50 individuals. Our correlation's with $p < 0.05$ were on average 0.087 ± 0.003 s.e.m, which is factor 5.7 smaller than their significant R_{ij}. In another study on *Z. marina* in the Falkenstein population Reusch et al. (1998) detected no significant linkage. There the highest value of R_{ij} was 0.142, which is still factor 1.6 greater than the average R_{ij} with significant linkage in this study. We would argue that in the present study we were able to

detect even weakest linkage between alleles because of the relatively large sample size (F_1 = 104; F_2 = 94; M_1 = 121; M_2 = 203) and hence high statistical power on the permutation tests. We therefore expect the effect of linkage on the assignment probability of clone identity to be small.

Mapping of density and flowering

In addition to the genetic mapping we counted the number of vegetative and flowering shoots in 40-cm x 40-cm sub-quadrates around each grid-point between Juli - August 2000, to obtain an estimate of shoot density and reproductive output m^{-2} $genet^{-1}$ for the sampled areas. In *Z. marina* flowering shoots are produced once a year and they can be distinguished from vegetative shoots in an early stage of development. Data from an eelgrass population in Denmark (c. 100 km from our site) showed that the appearance of new flowering shoots levels off in June and that the turnover is low during the summer month (Olesen, 1999). This could be confirmed with a random subset of 10 sub-quadrates in each plot that were re-measured after 1 month. No significant change in flowering intensity could be observed during this period (paired t; all $p > 0.13$). We thus assume that our single census is indicative of reproductive investment during the entire season.

Response variables and statistical analysis

We quantified multilocus heterozygosity as the proportion of typed loci for which an individual clone was heterozygous. Other studies (Coulson et al., 1998; Slate et al., 2000) have used other measures of heterozygosity such as mean d^2, or corrected mean d^2, which take the actual base pair difference between microsatellite alleles into account. These measures were not considered in the present study for two reasons: i) we were most interested in information on recent matings between relatives which is better reflected by individual heterozygosity (inbreeding depression), whereas mean d^2 is expected to measure events deeper in the pedigree (heterosis) (Pemberton et al., 1999) and ii) any interpretation of d^2 is based on the assumption of the stepwise mutation model, which may not hold. Another measure, standardised heterozygosity, calculated as the ratio of the heterozygosity of an individual to the mean heterozygosity of those loci at which the individual was typed, has been used to avoid potential bias that may be introduced by individuals being untyped at particular loci (Coltman et al., 1999; Slate et al., 2000). In the present study all samples included in the analysis were typed for all 9 loci, and therefore, standardised heterozygosity would have yielded the same results.

Both replicate plots in each population were very similar in terms of the measured traits (see results). Hence, data was pooled for each population for the analysis of flowering output and individual heterozygosity. For the correlations of flowering output, variables were transformed to achieve normality. To examine the association between heterozygosity and genet size we used two different approaches: 1) we followed a generalised linear models (GLMs) approach used in Coltman et al. (1998), Coulson et al. (1998) and Slate et al. (2000). Genet size was left-skewed and thus was modelled with a Poisson error structure and a log-link function was used for the GLM. The deviance ratio yields a χ^2-statistics with 1 df. 2) Because of the skewed frequency distribution across heterozygosity levels and because the 1 m^2 size-class was overpresented, we did a randomisation test of clone area and heterozygosity. From each randomisation (1000 in total) we calculated the mean area for each level of heterozygosity. From these means, simultaneous 95% confidence intervals were calculated with a procedure implemented in the software package GENSTAT which closely follows the methodology described by Hsu (1996). The formation of these intervals corrects for the number of intervals formed and takes into account the different numbers of observations per mean. The observed mean values were then compared to 95% confidence envelopes generated under the hypothesis of random pairing between clone size and individual heterozygosity. A significance level of $p < 0.05$ was used for all statistical tests. For data preparation (e.g. clone assignment) we used MATLAB 6.12. codes (Math Works, 2000) and for the statistical analysis GENSTAT 5 (Payne, 1997).

Results

Genet size-distribution and population comparison

The different disturbance susceptibility and frequency in the two sampled populations is reflected in large differences in the size distributions of their clones (Fig. II.2). In Falkenstein 27% of the area was covered by clones > 13 m^2 while in Maasholm no clone reached this size. In Maasholm clones of = 1 m^2 (1 sample) covered 60% while the same size - class accounted for only 30% of the covered area in Falkenstein. The distribution between the 1 m^2 and 13 m^2 size-class was similar for both populations. Accordingly, the fraction of area covered by large clones in Falkenstein is covered by the smallest clones in Maasholm. The high proportion of small clones found in the Maasholm population is reflected in the higher clonal

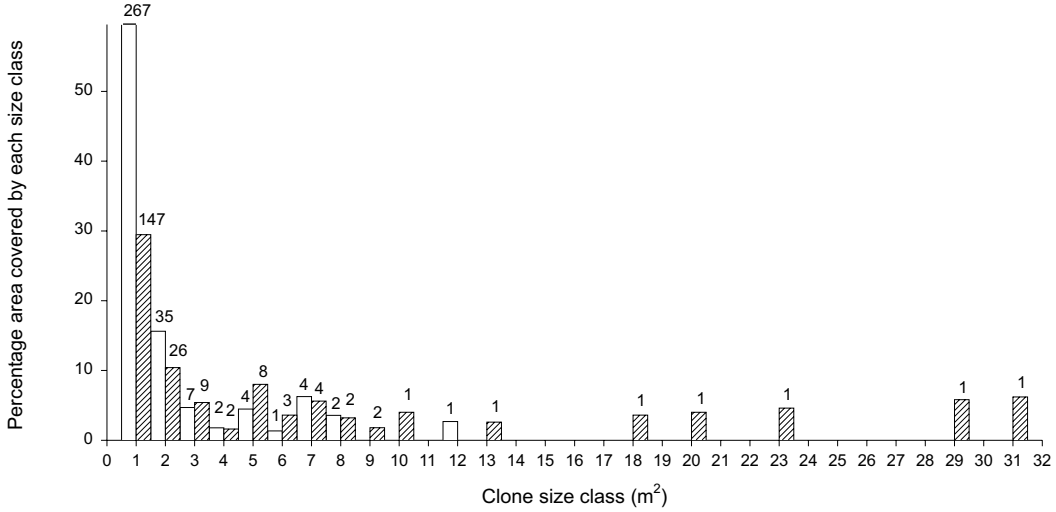


Fig. II.2: Percentage area covered by clones of each size-class in two *Zostera marina* populations, Falkenstein (hashed bars) and Maasholm (white bars) with number of observations on top of each bar.

diversity relative to Falkenstein (Table II.1). Further differences between the two plot pairs were the lower mean number of vegetative and flowering shoots and the lower flowering percentage in Maasholm compared to Falkenstein. In Maasholm a higher number of grid-points was non-vegetated indicating physical disturbance. Moreover, a higher number of distinct multilocus genotypes was detected as compared to Falkenstein. The allele distribution and the mean level of heterozygosity were similar in both populations.

Clone-area and flowering intensities

There was a significant positive correlation between the area of a clone and the number of flowering shoots counted within a clone for Falkenstein r = 0.50 ($F_{1,208}$ = 71.18; p < 0.001) and Maasholm r = 0.34 ($F_{1,321}$ = 44.2; p < 0.001) (Fig. II.3a,b) as would be expected from a relatively homogeneous distribution of flowering shoots throughout the sampled areas. Hence, the total reproductive output increased with clone size. The correlations between area of a clone and mean percentage flowering shoots within a clone were not significant both for Falkenstein r = 0.04 ($F_{1,208}$ = 1.38; p = 0.178) and Maasholm r = 0.00 ($F_{1,321}$ = 0.75; p = 0.388) (Fig. II.3c,d). This indicates that the relative investment into sexual reproductive output does not change significantly across the observed size range of clones.

Table II.1: Number of alleles ($N_{alleles}$) for each of the 9 microsatellites typed in this study (GenBank accession numbers 249303 - 249307, 009898, 009900, 009901 & 009904). The loci were amplified in a combined 4-primer PCR with 24 cycles (A) and one combined 5-primer PCR with 28 cycles (B). Also given are total number of alleles, number of samples typed for all 9 loci, number of empty grid points (non-vegetated space at time of sampling), number of distinct multilocus genotypes detected, genotypic diversity ($P_D = N_{genets} / N_{samples}$ (9 typed); (Ellstrand, Roose, 1987)), mean heterozygosity, density, flowering and flowering % for two plot pairs within two populations (Falkenstein & Maasholm) along the German Baltic Coast.

		Falkenstein		Maasholm	
		F_1	F_2	M_1	M_2
(A)	$N_{alleles}$ ZosmarCT-3	12	10	11	12
	$N_{alleles}$ ZosmarCT-17H	15	14	15	17
	$N_{alleles}$ ZosmarGA-2	6	7	9	7
	$N_{alleles}$ ZosmarCT-35	23	24	19	20
(B)	$N_{alleles}$ ZosmarCT-12	8	4	4	3
	$N_{alleles}$ ZosmarCT19	3	2	4	3
	$N_{alleles}$ ZosmarCT-20	6	8	4	5
	$N_{alleles}$ ZosmarGA-3	5	6	7	9
	$N_{alleles}$ ZosmarGA-6	8	6	5	9
	$N_{alleles}$ total	86	81	78	85
	$N_{samples}$ (9 typed)	246	253	209	239
	$N_{samples}$ (empty)	1	3	43	10
	Ngenets	109	101	122	201
	P_D	0.443	0.398	0.584	0.841
	Heterozygosity	0.48 ± 0.014	0.50 ± 0.014	0.45 ± 0.012	0.48 ± 0.009
	Density m^{-2}	347 ± 19.91	337 ± 53.28	277 ± 32.12	141 ± 19.91
	Flowering m^{-2}	31 ± 5.076	16 ± 2.273	4 ± 1.065	2 ± 0.237
	Flowering%	4.90 ± 0.500	4.40 ± 0.500	3.00 ± 1.200	2.20 ± 0.600

Clone-area and individual heterozygosity

Individual heterozygosity explained significant variation in clone size in Falkenstein (χ^2_1 = 38.41, $p < 0.001$) but not in Maasholm (χ^2_1 = 0.07, $p = 0.78$). Relatively outbred clones in Falkenstein were on average larger (Fig. II.4a). No genotypes with heterozygosities < 0.55 were contributing to the very large clone size-classes (> 10 m^2). The randomisation test showed that the mean area of genets significantly deviates from random expectations for both populations (Fig. II.4c). The mean area in Falkenstein was significantly lower than expected from random pairings for levels of heterozygosities at 0.33 and 0.44 and significantly higher for levels of heterozygosities at 0.55 and 0.66. This supports the positive correlation from the GLM for Falkenstein. For Maasholm, only the value for mean area at 0.55 fell above the 95% confidence limit, and the value at 0.66 fell below the 95% confidence limit, indicating a similar

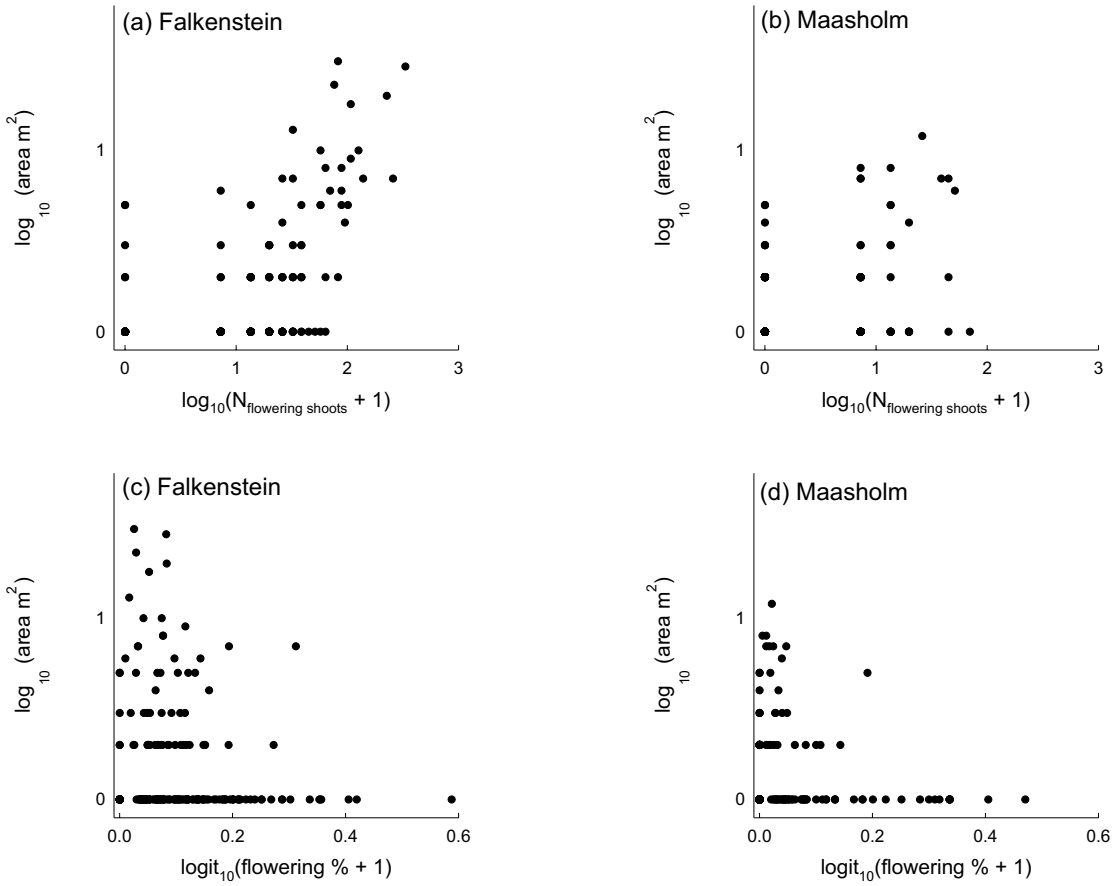


Fig. II.3: Correlations of area with the number of flowering shoots in clones from Falkenstein (a) and Maasholm (b) and correlations of area with percentage flowering in clones from Falkenstein (c) and Maasholm (d).
Note that $\text{logit}_{10}(x) = \log_{10}(x/(1-x))$

albeit weaker trend also at this site. In addition, the randomization test identifies the consistent decrease in mean area at the two highest levels of heterozygosity in both sampled populations (Fig. II.4a,b). In both plots, mean clone area at the highest levels of heterozygosity was not higher than would be expected from random matching. It was even significantly lower for one mean value in Maasholm. However, the broad confidence envelopes at both ends of the range of heterozygosities indicate the low number of observations in these corners and hence the low statistical power.

The mean heterozygosities were 0.49 for Falkenstein and 0.47 for Maasholm. Fis values for the group of large clones (> 13 m^2) revealed a slight heterozygote deficit (- 0.15), yet no significant deviation from HWE could be observed (95% confidence limits from 1000 bootstraps).

Discussion

Based on a fine scale mapping of genets we have demonstrated that clone size increases with individual heterozygosity in the Falkenstein *Zostera marina* population. Moreover, measurements on sexual reproductive output support the prediction that clone size correlates with fitness. This suggests that the relatively outbred individuals in the meadow are occupying larger areas, because they are stronger competitors than their relatively inbred counterparts. Hence a deterministic process, namely inbreeding depression affects the size distribution of genets at a low disturbance site.

In the disturbance prone population of Maasholm the effect was similar although much weaker. At this site, the size-class responsible for the strong positive association with individual heterozygosity in Falkenstein (clones > 11 m^2) was absent (Figs. II.2 & 4b). Waterbird grazing is known as an importance source of disturbance in eelgrass meadows (Laubhan, Metzner, 1999; Percival et al., 1996; Wilson, 1995). The Maasholm seagrass meadows provide feeding grounds for swan (*Cygnus olor*). These meadows are located in a shallower area than Falkenstein and with sometimes significant changes in the water level (Marahrens, 1995). The different access to eelgrass in the two populations is reflected in the higher number of moulting and resting swan recorded for the Maasholm region (300 - 500) as compared to the Kiel Förde (50 - 100) of which Falkenstein is only part of (Erfurt & Dierschke,

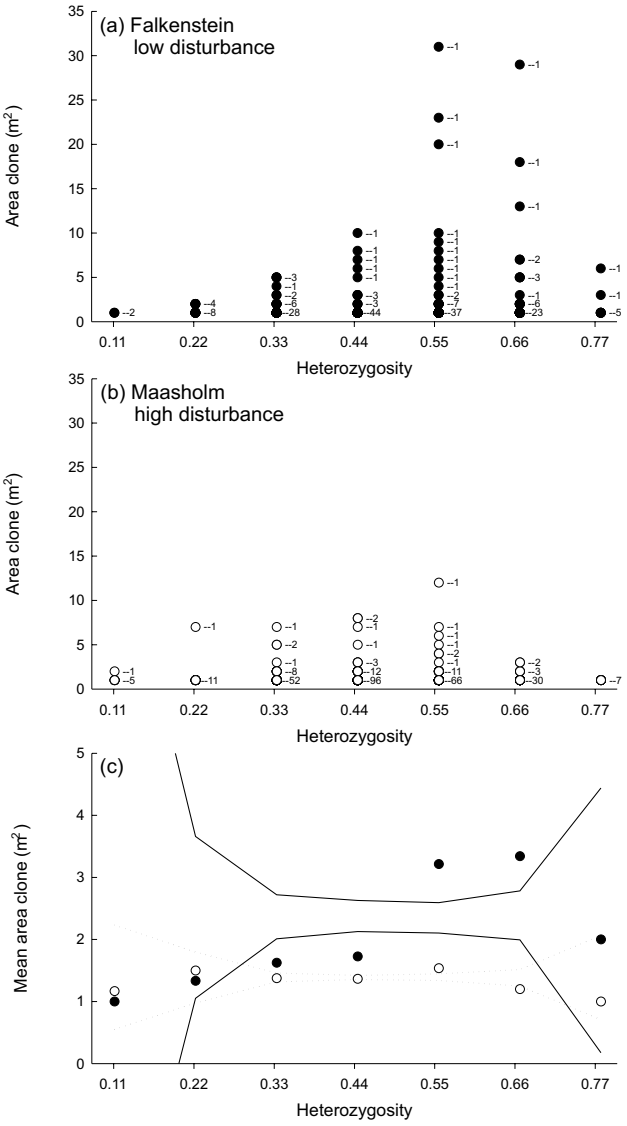


Fig. II.4: Clone area plotted against individual heterozygosity for clones from Falkenstein (a) and Maasholm (b). Because there are overlaying points in Figures (a) and (b) number of observations are given on the right side of each point. Mean area against individual heterozygosity is plotted in (c) for Falkenstein (filled circles) and Maasholm (open circles). The lines indicate 95% confidence envelopes for the hypothesis of random matching between clone size and heterozygosity for Falkenstein (solid lines) and Maasholm (dotted lines).

1992; Koop, 1996 & A. Ehlers personal communication). In addition to swan grazing, the impact of ice scour that occur every 8 - 10 yrs is likely to have a greater effect on the Maasholm meadows because their impact increases towards the shallower coastal areas. The absence of large clones in Maasholm and the gaps recorded in the canopy most probably reflect this high disturbance frequency. Selection on dominant clones in terms of spatial expansion can only take place if clones have enough time to outcompete weaker genotypes.

Associations between multilocus heterozygosity and fitness components are common place (Britten, 1996; Mitton, 1998). The statistical power for detecting these associations is usually low and many negative results go unreported (Rowe & Beebee, 2001; Whitlock, 1993). In addition, most studies investigating heterozygosity and fitness in plants were either conducted under controlled laboratory conditions (Waldmann, 2001), involved breeding strains (Kimbeng & Bingham, 1998) or compared populations (Fisher & Matthies, 1998; Madsen et al., 2000; Mccall et al., 1991; Oostermeijer et al., 1998; Raijmann et al., 1994) rather than individuals (but see Bush & Smouse, 1991; Holtsford & Ellstrand, 1990; Strauss, 1987). For clonal plants we found only one other study reporting an increase in clone size with individual heterozygosity for the clone forming canyon live oak (*Quercus chrysolepsis*) based on allozyme loci (Montalvo et al., 1997). The fitness aspect of clone size was however not explored.

It is well possible that several other studies using traditional codominant markers (e.g. allozymes) did not detect a heterozygosity-fitness relationship because the used markers showed low variability (slow mutation rate) together with selection on some of the loci. For example, in a study on allozyme variation in a Danish population of Armeria maritima, plant diameter was used as a measure of plant age. Yet, no significant association between plant diameter and individual heterozygosity was found (Weidema et al., 1996).

The self compatible *Z. marina* is known to suffer inbreeding depression after selfing. Ruckelshaus (1995) found in pollination experiments that a greater proportion of outcrossed flowers set seed as compared to selfed flowers. This is supported by the finding that deselection of selfed progeny took place after geitonogamous matings in field populations (Reusch, 2001), effectively restoring HWE-proportions in the adult population. The results of this study suggest selection on relatively outbred clones also in later stages of clone life-history. Dispersal distances for *Z. marina* are short (Ruckelshaus, 1996) which limits interactions between individuals to the local neighbourhood (Stoll & Weiner, 2000). In the Baltic sea *Z. marina* forms almost monospecific stands in a relatively homogeneous abiotic environment over short distances (1 - 10 m). Hence the local neighbourhood of an eelgrass clone consists of conspecifics, competing for the same space. It is possible that mainly competitive interac-

tions are responsible for the deselection of inbred clones in the course of spatial expansion, although there is no concrete evidence for this.

The positive correlation between clone size and the number of flowering shoots is comparable with the finding of Handel (1985) who found a positive correlation between genet size and the number of culms in the clonal woodland sedge *Carex platyphylla*. However, in particular in self compatible clonal plants with abiotic pollination (water or wind) such a positive correlation may be modulated by two additional processes. (i) Large clones will always have a greater proportion of endogenous pollen landing on their stigma than do smaller clones and (ii) for flowering shoots with central positions within large clones this effect will even be stronger because of the limited access to foreign pollen. This means that the reproductive output and the fitness of offspring will be negatively affected by increased between-flower selfing (geitonogamy) with increasing clone size (Eckert, 2000). It has been predicted that genet size is a fitness correlated trait (Gardner & Mangel, 1999) and the positive correlations from our results support this prediction. It is clear however, that many factors influence life-time clonal fitness and that the shape of the fitness curve with clone size is certainly more complex than the almost linear increase of flowering output with size suggests (Fig. II.3a,b).

Clonal plants are expected to show senescence in terms of a decline in the rate of sexual reproduction with clone age (~ size) (Gardner Shea & Mangel, 1997). In this study clone size was not significantly correlated with percentage flowering which is indicating that at least for the observed size-range genets do not loose or gain vigour in reproductive output with increasing size. Hence, there is no indication for senescence in the rate of sexual reproduction. It is well possible that in northern latitudes where *Z. marina* has a lower turnover rate and single clones can cover much larger areas (Reusch et al., 1999) senescence in terms of decreasing flowering is present and contributes to the existence of very large clones (> 50 m^2).

Unexpectedly, we found a consistent decline in mean area at the two highest levels of heterozygosity in both sampled populations (Fig. II.4), which provides an interesting starting point for future studies. If low levels of individual heterozygosity indicate inbreeding depression, then at high levels of individual heterozygosity we may observe outbreeding depression with the highest values of fitness components around an optimal level of heterozygosity. Optimal outcrossing has been demonstrated for several plant species by pollinating target individuals with pollen from donors of varying distance to the target (Irwin, 2001; Mccall et al., 1991; Price & Waser, 1979; Schierup & Christiansen, 1996; Trame et al., 1995; Waddington, 1983; Waser & Price, 1989; Waser & Price, 1991; Waser & Price, 1994). This is the first marker based study indicating a loss of fitness at very high individual heterozygosity levels. In prin-

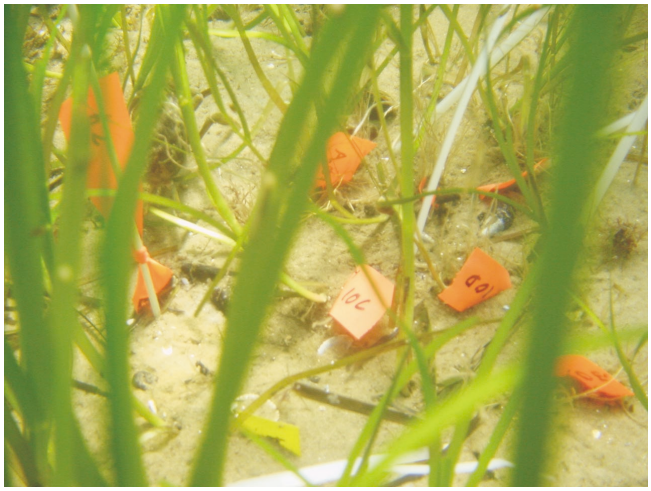
cipal we should expect a negative effect of heterozygosity on fitness components at both low and high levels of heterozygosity (Charlesworth & Charlesworth, 1999) and possibly our data is indicating such a phenomena. Unfortunately and unavoidably the statistical power at both ends of heterozygosity levels is very low because of the few observations. Future studies aiming at investigating optimal outcrossing with a molecular approach will require large sampling efforts. In addition, it would be helpful to have some predictions on the shape of the curve describing optimal outcrossing rather than inbreeding depression alone.

We have thus shown that clone structure is a function of deterministic and stochastic processes. The former have a large genetic component, as marker correlated genetic attributes of genotypes explain a significant proportion of the large size difference in clones.

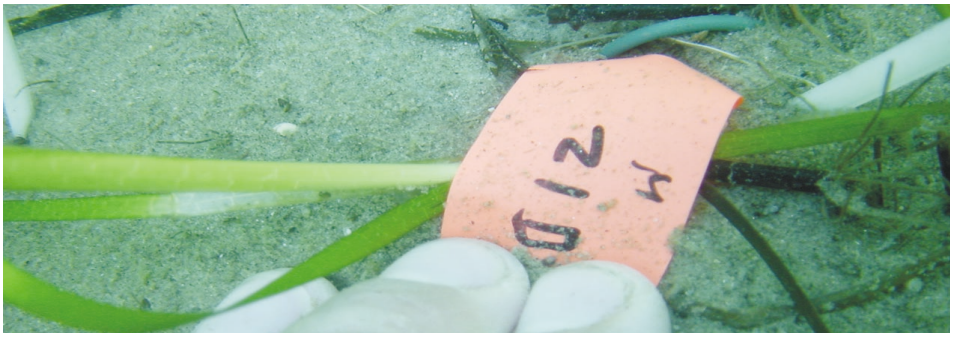
Acknowledgements

We like to thank Anneli Ehlers and Sascha Romatzki for helping in the field, Silke Carstensen and Catha Schmuck for help with the fragment analysis and Annelis Lüscher for comments on an earlier draft of this manuscript. This work has been funded by a grant to Thorsten Reusch from the Deutsche Forschungsgesellschaft (DFG), Grant No. 1108/3-1 and 3-2.


Chapter - III



Transplantation patch in the Falkenstein meadow



Checking the tag of a transplanted eelgrass shoot, anchored with a wire peg.

Local adaptation and transplant dominance in genets of the marine clonal plant *Zostera marina*

Summary

Worldwide decline of seagrass beds has brought up the need for information on potential source populations for re-colonisation and conservation. The aim of the present study was to test for local adaptation in *Zostera marina*, the dominant seagrass species in the northern temperate zone. To this end we performed a reciprocal transplant experiment at the level of the genetic individual in two Baltic Sea populations for which the clonal structure had been mapped in detail. The treatment effects were tested on above-ground and below-ground dry weight of physiologically independent rhizome fragments of replicated genets at the end of the season. We found that i) genets from both populations produced more biomass in their home population (local adaptation), ii) genets from one population produced more biomass overall (overall dominance) and iii) the range of clonal performance was indicating a high degree of variability among genets within the two populations. Our results provide a first test for local adaptation in established seagrass genets and demonstrate home site advantage of clones that are part of a highly connected system of *Zostera marina* populations along the Baltic Coast.

Introduction

Seagrass meadows rank amongst the most productive ecosystems in the world (Costanza et al. 1997), yet they are threatened by large scale declines during the past decades (Orth & Moore 1983, Giesen et al. 1990, Short & Wyllie-Escheverria 1996). In this context it has become increasingly important to have information on the suitability of plants from potential donor populations for transplantation and re-colonisation purposes (Fonseca et al. 1998, Van Katwijk et al. 1998, Williams 2001). One important factor to consider in re-colonisation efforts is the ubiquitous presence of local adaptation in naturally occurring plant populations. Being sessile with a limited seed and pollen dispersal, genotypes often reveal reduced fitness in a foreign environment (Antonovics 1971, Antonovics et al. 1971). Alternatively in clonal plants, genets may be multipurpose genotypes which perform well under many habitat conditions (Lynch 1984).

While several studies have looked at factors influencing transplantation and recolonisation success in seagrasses (Dennison & Alberte 1986, VanLent & Verschuure 1995, Van Lent et al. 1995, Van Katwijk et al. 1999, Pranovi et al. 2000, Procaccini & Piazzi 2001) still surprisingly little is known about the degree of local adaptation in marine angiosperms. For example, Van Katwijk et al. (1998) and Van Katwijk & Hermus (2000) have looked at depth limitation and differential success to an artificially created light gradient in a mesocosm experiment in eelgrass (*Zostera marina*) in the Wadden Sea. Worm & Reusch (2000) found initial shoot density in planted patches to have a positive effect on recolonization in eelgrass in the Baltic Sea. Procaccini & Piazzi (2001) have found that growth and rhizome branching rate of transplants of *Posidonia oceanica* was positively associated with the level of genetic diversity in the source population. Most of these studies involved transplantation in one direction and across replicated ramets rather than genets.

The aim of the present study was to test for local adaptation in clones of eegrass (*Zostera marina*), the dominant seagrass species of the northern temperate zone (Den Hartog 1970). To this end we performed a reciprocal transplant experiment with a total of 20 genotypes from two populations on the Baltic Coast, for which the genet structure had been mapped in detail. The experiment closely followed a design suggested by Via (1994). The interaction between source site and transplantation site is testing the prediction that in the case of local adaptation, genets from the two source sites have significantly different relative performance in the two transplantation sites. Because we were interested in local adaptation at the level of the genetic or evolutionary individual (*sensu* Janzen 1977) we replicated genets rather than populations. The transplants were physiologically independent rhizome fragments

from single genets, as they can be found in the field (Watson & Casper 1984, Callaghan et al. 1992), which we call fragments. This is in contrast to previous studies where transplants were either single shoots or ramets, representing potentially independent units of a clone (obtained by breaking the rhizome) instead of structures occurring in natural populations.

Methods

The study system

In the non tidal Baltic sea, *Zostera marina* is a long-lived perennial and forms meadows with clones of variable size and age. In established meadows with closed canopy, seedling recruitment is rare (Robertson & Mann 1984, Olesen & Sand-Jensen 1994b, a & pers. obs.) Thus, open space is mainly colonised through branching and the horizontal growth of rhizomes. The transplant experiment involved two sites along the German Baltic Coast separated by c. 50 km of coastline, each home to a natural population of *Z. marina* growing in 2.5 m - 3.5 m water depth. The 'Maasholm' population is situated in the estuary at the river Schlei, Schleswig Holstein (54°41' N, 10°00' E). The 'Falkenstein' population grows on the west side of the Kieler Förde, Schleswig Holstein (54°24' N, 10°12' E). Both sites belong to the Kiel Bight water body and therefore have similar salinity and temperature regimes (Reusch 1998). However, the slightly shallower Maasholm population is more prone to disturbance by swan grazing and ice scour than the Falkenstein population (personal obs.).

Mapping of genets

In order to obtain plants with known genetic identity, high resolution clonal mapping was conducted in the field. During the month May and June 2000, two 15-m x 15-m permanent plots were set up in shallow water eelgrass beds in the two above mentioned populations, using SCUBA. The plots were sampled on a 1-m regular grid (a total of 1024 samples). Leaf material of plants closest to the grid points was collected and preserved in silica-gel for DNA amplification. The DNA was extracted with the Qiagen plant DNA extraction kit (Qiagen, Hilden, Germany). The DNA-extract was processed in a combined PCR-amplification using the four most polymorphic microsatellite markers (GenBank accession no. AJ249307, AJ249305, AJ009900, AJ009898) specifically designed for *Zostera marina*, with fluorescent labels. The

genotyping followed standard protocols (Reusch et al. 2000). Alleles were scored on an ABI 377 automated sequencer, using the software packages GeneScan 3.1 and Genotyper 2.0 (Biosystems 1998). This produced 256 pixel images of the genet composition in the plots. Because the two sampled plots within each population were very similar in terms of clonal diversity (number of distinct multilocus genotypes/number of samples) and genet size distribution they seemed to be representative for the population structure in this area. In addition to the genetic mapping, 40-cm x 40-cm sub-quadrates around each grid-point were counted for number of shoots, in order to obtain an estimate of shoot density m^{-2} $genet^{-1}$ for the sampled areas.

Experimental set-up

We tested for local adaptation in genets of *Z. marina* with a reciprocal transplant experiment between the two populations Falkenstein and Maasholm. In the literature the term 'ramet' is usually reserved for a potentially independent module in a clonal plant. In this work we addressed the actually independent rhizome fragment of a genet found in the field (Watson & Casper 1984, Callaghan et al. 1992) which we call 'fragment'.

From 15-m x 15-m plots in Falkenstein and Maasholm that had been mapped for clone structure, ten genets of all genets with ≥ 3 m^2 area (n= 23) were randomly selected. Between 17 and 19 Mai 2001 from each of these genets, 10 fragments were excavated and stored in tanks of fresh sea water for transportation. Special care was taken not to break rhizomes other than within the rotten parts, in order to obtain effectively independent units rather than potentially independent units. Each plant was individually marked with a plastic dented tide (Solana-Arellano et al. 2000) and measured for shoot and rhizome length. Mean rhizome length (cm) of fragments for each treatment level was: for Falkenstein, back-transplants 6.57 ± 0.72, transplants 6.03 ± 0.43 and for Maasholm, back-transplants 6.62 ± 0.47, transplants 6.67 ± 0.60. Each fragment had 1 - 3 shoots. Five fragments of each genet were randomly assigned to one of the sites, either back to the source site or to the transplantation site (Fig.III.1). All sediment was removed from the rhizome to reduce carryover effects. On 17 and 19 May, fragments were planted in groups of five into a randomly chosen genet from where plants (same genet or different genet) had previously been removed. With the aid of a small shovel, rhizomes were lowered into the soft sediment and gently anchored with garden wire pegs. It is clear that it would have been ideal to have a mesocosm stage before transplanting the fragments to reduce possible maternal effects (Futuyma 1986). Maternal effects may result from genetic and/or environmentally based differences among maternal phenotypes.

For example a classic study of maternal effects in mice (Falconer 1965) found that large mothers had many small young. The lack of resemblance between mothers and their daughters in litter size and offspring size was mediated by the phenotypic effect of maternal size and its relation to maternal provisioning ability. In a study on the clonal plant Solidago altissima Schmid & Dolt (1994) found that the mean dry mass of seedlings was significantly influenced by both the maternal genotype and the maternal environment.

Maternal effects in plants seem to affect mainly early life history stages (seeds, seedlings) (Schmid & Dolt 1994, Thiede 1998) and appear to be small compared to the effects of genotype and environment (Weiner et al. 1997). Because we were using fragments of well established adult genets and not seeds and seedlings, we assumed such effects to be small for the measured variables.

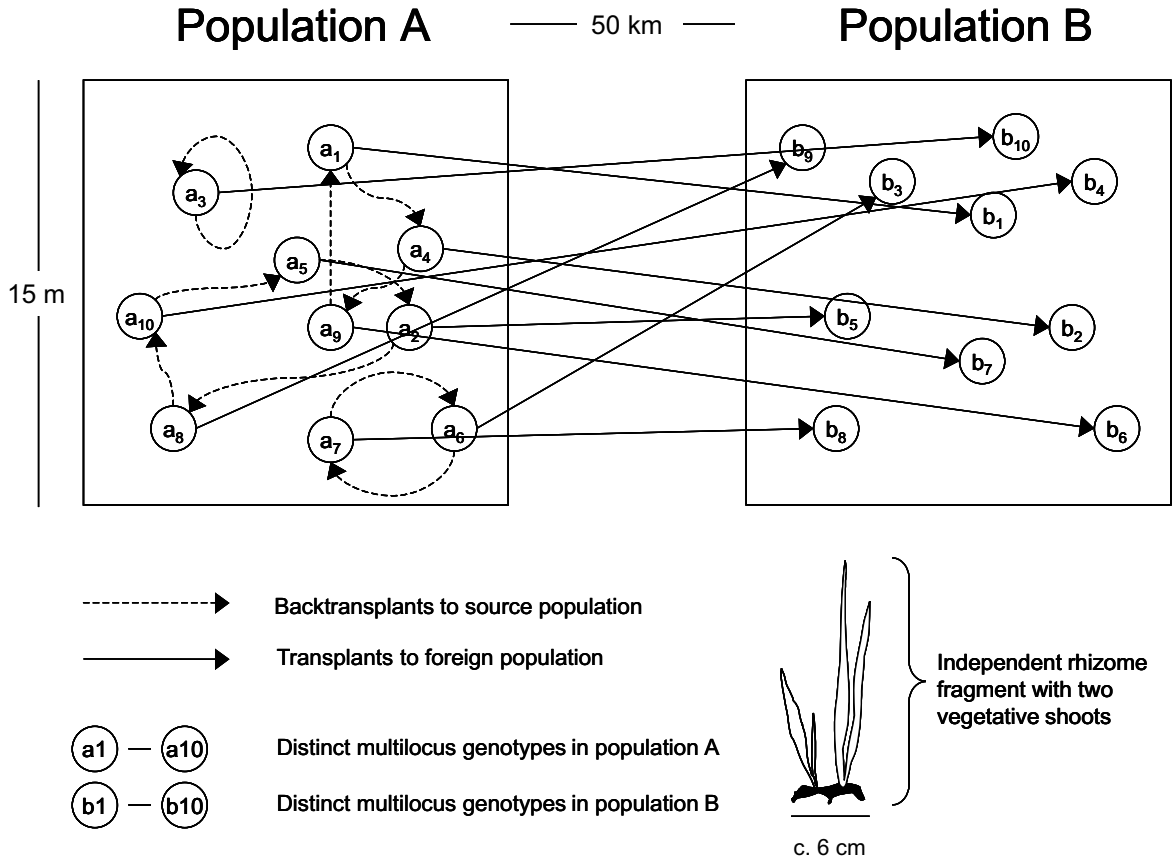


Fig. III.1: Experimental set-up to test for local adaptation in genets of eelgrass (*Zostera marina*) between 15-m x 15-m plots in Falkenstein and Maasholm along the Baltic Coast. Transplantations and back-transplantations are indicated with arrows. Each arrow indicates the movement of 5 independent rhizome fragments of which one example is shown in the lower right corner of the figure. For reasons of clarity, the transplantation scheme only includes 4 instead of the actual 10 randomly chosen genets in each plot. Please note that allocation of sites (source site or transplantation site) was random, and therefore both cases, transplantation back to the original genet and transplantation into different genets within the same site could occur.

The experiment lasted 11 weeks, from the beginning of the growth phase in spring, to seed ripening. Of a total of 200 fragments, 156 fragments (78 %) could be recovered and harvested on 1st and 2nd August 2001. The time of harvest was chosen such that flowering shoots had not yet started to detach from their rhizome and hence would have been lost for the above ground dry weight measurements. At that time those fragments bearing flowering shoots (36 out of 156), in addition to the vegetative shoots, had produced seeds. A random sample of an additional 20 fragments per source site was harvested one week later, in order to obtain measurements from non transplanted controls as reference points from each location. Fragments were transported to the lab in water tanks, stored in Zip lock bags at 4 °C and then measured and prepared for drying.

Fragments were separated in above-ground and below-ground biomass by cutting off the shoots above the first root bundle. Epiphytes were removed manually with a tissue paper. Above-ground and below-ground biomass was determined to the nearest 0.01 g after drying at 80 °C to a constant weight.

Statistical analysis

The ANOVA model for genet means contained two treatment factors, 1) source site (fragments were taken from Falkenstein or Maasholm (N = 20 genets)) and 2) transplantation site, (fragments were transplanted back to source site or to foreign site (N = 20 genets)), and one random factor (distinct multilocus genotype). Response variables were below-ground and above-ground biomass. All analysis were done with the software package GENSTAT (Payne 1997).

Fragments from each of the 10 genotypes in both source sites (N = 20 genets, 5 fragments per genet) were randomised within the sites. At the time of planting, rhizome length and shoot height where not significantly different between the treatment levels (F was always < 2 and p always $>> 0.05$). The reference points (i.e. non-transplanted controls) for shoot and rhizome dry weight were tested against a random sub-sample of 20 fragments from the back-transplanted fragments in each population using unpaired t-tests.

Results

Shoot dry weight was positively correlated with shoot length r = 0.842 ($F_{1,145}$ = 383; p < 0.001) and rhizome dry weight was positively correlated with rhizome length r = 0.628 ($F_{1,145}$ = 102.5; p < 0.001). Note that the correlation with shoot dry weight and length was only calculated for terminal shoots but is similar for additional shoots. These correlations indicate that dry weight is a good indirect measure of plant size (data not shown).

Mean above-ground dry weight of genets differed significantly between the two source populations Falkenstein and Maasholm ($F_{1,18}$ = 12.95; p = 0.02) and there was a significant interaction between source site and transplantation site ($F_{1,18}$ = 9.69; p = 0.006), the test for local adaptation (Fig. III.2a). Plants from Falkenstein yielded more above-ground biomass in both populations. Back-transplants in Falkenstein clearly outperformed plants from Maasholm while transplants from Falkenstein to Maasholm attained similar values as the resident back-transplanted genets in Maasholm. Below-ground dry weight measurements did not differ significantly between Falkenstein and Maasholm at the 5 % level ($F_{1,18}$ = 3.28; p = 0.087) but again there was a significant interaction between source site and transplantation site ($F_{1,18}$ = 5.31; p = 0.033) (Fig. III.2b), indicating local adaptation. Back-transplants in Falkenstein outperformed Maasholm plants in terms of rhizome biomass yield while transplants from Falkenstein to Maasholm attained similar values as the resident back-transplanted genets in Maasholm.

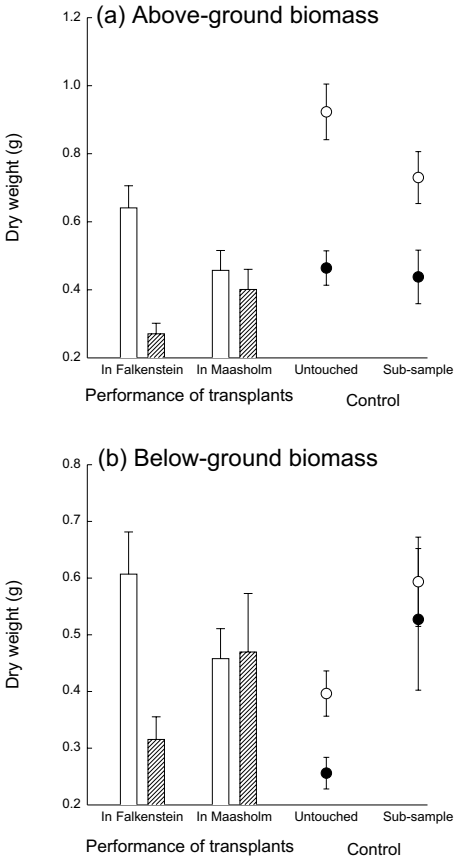


Fig. III.2: Mean dry weight (± 1 SE) for above-ground (a) and below-ground (b) biomass for genets from Falkenstein (white bars) and Maasholm (hashed bars) reciprocally transplanted between the two source sites (means over 10 genets). Single points on the right side represent random samples of untouched (untransplanted) controls for Falkenstein (open circles) and Maasholm (closed circles) and random sub-samples of back-transplants from Falkenstein (open circles) and Maasholm (closed circles) against which the untouched controls were tested (means over random samples of 20 independent rhizome fragments).

For above-ground biomass, there was no significant difference between non-transplanted controls and a random sample of 20 back-transplanted fragments, (Falkenstein: $t_{1,38}$ = 1.72; p = 0.093; Maasholm: $t_{1,38}$ = -0.28; p = 0.784). However, non-transplanted controls showed significantly lower values for rhizome dry weight in both source sites (Falkenstein: $t_{1,38}$ = 2.23; p = 0.031; Maasholm: $t_{1,38}$ = 2.12; p = 0.041) (Fig. III.2a,b).

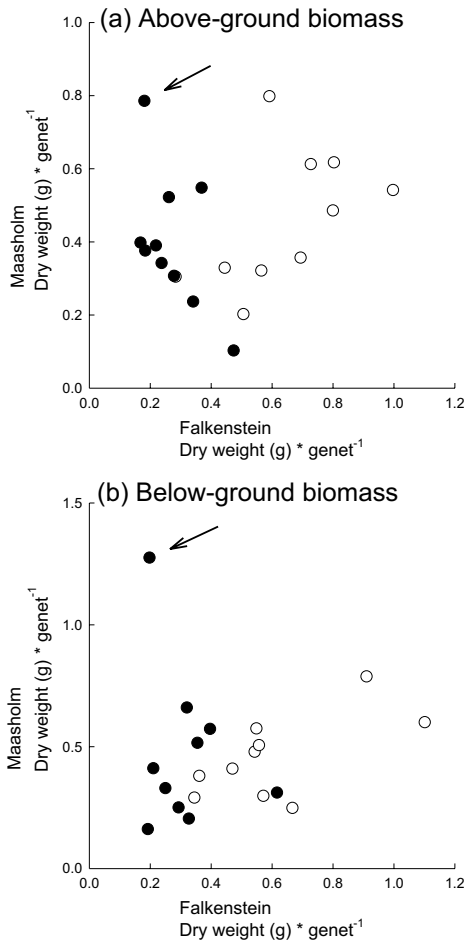


Fig. III.3: Range of genet performance in the two sites for above-ground (a) and below-ground (b) biomass for clones from the two source sites Falkenstein (open circles) and Maasholm (closed circles). The values were obtained by taking the mean over all back-transplanted fragments (≤ 5 rhizome fragments) of each of the 20 genets (x-values for Falkenstein & y-values for Maasholm) and the mean over all transplanted fragments (≤ 5 rhizome fragments) of each of the 20 genets (y-values for Falkenstein & x-values for Maasholm). The arrow indicates an outlier (see text for explanation).

Table III.1. Number of genets (N_{genets}), clonal diversity as $N_{genets}/N_{samples}$ ($C_{diversity}$), total number of alleles ($N_{alleles}$), mean (± 1SE) percentage of heterozygote loci (Heterozygosity) and number of shoots m^{-2} (Density), for two 15-m x 15-m plots within two *Zostera marina* populations, Falkenstein and Maasholm, along the Baltic Coast.

	Falkenstein	Maasholm
N_{genets}	104	121
$C_{diversity}$	0.408	0.567
$N_{alleles}$	90	104
Heterozygosity	0.733 ± 0.02	0.717 ± 0.018
Density m^{-2}	347 ± 19.91	277 ± 32.12

Of the 156 harvested fragments, 36 were bearing flowering shoots. This corresponded closely to the natural level of fragments bearing flowering shoots (Hämmerli & Reusch unpub. data), as could be expected from a random sampling. A separate analysis of reproductive biomass did not change the results for local adaptation.

Average values per genet and site gave an indication for the range of clonal performance (Via 1994) for the 10 genets from each source population (Fig. III.3 a, b). One back-transplanted genet from Maasholm was found growing in an open site at the time of harvest. At the time of planting this patch of meadow had been located close to an erosion front that had moved during the summer and cleared most vegetation by the end of the season leaving the remaining back-transplants growing in a gap. This value contributed much to the overall variance and was considered an outlier (marked with an arrow in Fig. III.3 a,b). Cross clone correlations were calculated both with and without this outlier for comparison. The correlation coefficients with outlier were r = 0.221 ($F_{1,19}$ = 0.944; p = 0.344) for above-ground and r = 0.114 ($F_{1,19}$ = 0.235; p = 0.633) for below-ground biomass, respectively. Excluding the outlier, we found r = 0.401 ($F_{1,18}$ = 3.261; p = 0.088) for above-ground and r = 0.493 ($F_{1,18}$ = 5.499; p = 0.034) for below-ground biomass. Without the outlier there was a significant positive cor-

relation for below-ground biomass but this correlation was weak and not significant for above-ground biomass. Within the sampled plots we found a higher number of distinct multilocus genotypes, a higher genetic diversity (number of distinct genotypes / number of samples) and a higher total number of alleles in Maasholm as compared to Falkenstein. Shoot density m^{-2} $genet^{-1}$ and mean heterozygosity was not significantly different between the two populations ($t_{1,222}$ = 1.78; p = 0.077) and ($t_{1,222}$ = 0.6; p = 0.55) respectively (Table III.1).

Discussion

The significant interaction between source site and transplantation site is showing local adaptation in *Zostera marina* for genets from Falkenstein and Maasholm. Genets from both sites showed a higher relative performance in their home population. To our knowledge this is the first data on local adaptation of replicated genets in a seagrass species.

At the time of harvest genets from Falkenstein had twice as much biomass in their home population compared to transplants from Maasholm and they could keep up with the resident genotypes when transplanted to Maasholm. Since planted fragments had similar starting conditions this indicates that Falkenstein genets were more efficient in occupying space than genets from Maasholm. The reasons for such transplant dominance must remain speculative. Genotypic diversity was considerably higher in Maasholm, 39 % for the plots chosen for the experiment (Table III.1), which was contrary to the results of Reusch (2001) who found higher clonal diversity in Falkenstein, based on a smaller random sample of 25 - 46 genets from 1997, at slightly different locations. This does however not change the finding that the two populations are part of a network of populations that seem to be highly connected (Reusch 2001). In a common garden transplant experiment Procaccini & Piazzi (2001) found that plants from the population with the highest genotypic diversity (0.48, from table 3) and the highest level of heterozygotes were those with the highest overall rhizome growth and branching rate. In the present experiment the genets that could grow to a larger size during the season came from Falkenstein, the populations with the lower genotypic diversity and a level of heterozygosity not significantly different from Maasholm (Table III.1).

Leaving aside local adaptation, we found also a high degree of variability in the relative performance of genets in both foreign environments (Fig. III.3 a,b). Figure III.3 also shows the divergence between the groups of genets from the two source environments as expected from locally adapted populations (Via & Lande 1985, Via 1994) and variability among genets from the same source population. Furthermore, genets with a high biomass yield in one site

also achieved high biomass yields in the other site. These correlations were, however, only significant for the response variable “rhizome dry weight” (Fig. III.3b).

The fragments were transplanted into a closed leaf canopy in order to simulate the environment they presumably were adapted to as closely as possible. However, it was unavoidable that the planting spots had softer sediment and the rhizomes of the transplants were less entangled compared to plants in the pristine meadow. Possibly this slight reduction in local density decreased competitive interactions. Several studies have shown that low density in the neighbourhood can shift allocation from shoot to rhizome growth (Landhausser et al. 1996, Hubersannwald 1998, Van Kleunen et al. 2001). We suggest that similar processes explain differences between untouched controls and the back-transplants from both populations. Rhizome dry weight of back-transplants were consistently and significantly lower compared to the values from untouched controls (Fig. III.2b), suggesting that transplants experienced lower competition for below-ground space. Further support for this explanation comes from the sole genet from Maasholm growing in a vegetation gap at the time of harvest. It showed considerably higher values compared to the other genets from that population (outlier in Fig. III.3 a,b). Population differentiation might at least in part be due to different competitive regimes (Gurevitch 1986, Wilson & Keddy 1986). The observed differences in rhizome biomass between untouched controls and back-transplants in the present study could indicate that for *Z. marina* intraspecific interactions play an important role in local adaptation. Most probably the genetic neighbourhood in a meadow rather than abiotic components constitute the environmental patchiness for *Z. marina* clones.

The present study is a first step towards understanding local adaptation in seagrasses. Some limitations have to be considered carefully. Ideally, any detection of local adaptation should be based on home-site advantages measured by life time fitness. Such a goal is rarely feasible for long-lived clonally growing species such as *Z. marina* (Kindell et al. 1996). In *Z. marina* rhizome connections break over time (1-2 yr) leaving behind clusters of physiologically independent units that are all part of the same clone covering many m^2. However, future life-history stages not examined here would have to show a reversal of the differential performance found in the present study to cancel out local adaptation. This seems unlikely considering that we transplanted independent modules of well established genets. Including future stages (e.g. larger units) would probably affect the magnitude of the observed local adaptation rather than its existence.

Early life history stages such as seedling recruitment, although presumably rare in closed meadows (Robertson & Mann 1984, Olesen & Sand-Jensen 1994b, a), are an important but still poorly understood component of *Z. marina* population dynamics. Ruckelshaus

(1994) reports on an experiment that indicated local adaptation of seeds between different tide zones based on germination rates and seedling mortality. However, experiments on early stages are susceptible to possible confounding by maternal effects. For a more complete picture it will be necessary to follow early recruitment stages over a longer time period comparing establishment in open sites with establishment in the closed meadow in the context of local adaptation.

We were mainly interested in between site effects of local adaptation using replicated genets. In a conservation context this is the most relevant scale, as plants are often translocated from a donor to a target site. However, it would be interesting for future experiments to look more closely at the scale to which *Zostera marina* clones are adapted to (close neighbourhood (1-5 m), population level (6-1000 m)) with a design that differentiates between transplantation back to the original site and back to the home environment, but away from the original site, as suggested by Underwood (1990) and Chapman (2000).

Marker diversity is often used to decide which populations are most suitable as a source for translocation and restoration (Templeton 1986, Haig 1998, Knapp & Rice 1998), with the assumption that those possessing the greatest level of genetic variation are those with the greatest adaptive potential (Vrijenhoek 1994, Moritz et al. 1995). However, it has been shown recently that such a practice is not without risks (McKay et al. 2001) because plants from locally adapted populations that do not show marker diversity could still suffer from reduced fitness in a foreign environment. The two populations Falkenstein and Maasholm have similar genetic diversity and their genetic differentiation is small in absolute terms (θ only 0.02) (Bohonak 1999). Only after transplantation we find different relative performance of genets from the two source sites. Therefore it is important to support genetic data with studies on the variation of quantitative characters between populations. The results of the present study should contribute to this discussion.

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Chapter - IV

Rafting flowering shoot in Maasholm - on its way to mix up kinship structure in a new population?

PVC post, permanently marking the edge of an eelgrass plot.

Spatial autocorrelation of microsatellites reveals kinship structure in a marine clonal plant

Summary

Limited dispersal distances in plant populations are leading to local genetic structuring, which can be examined with spatial autocorrelation techniques. In clonal plants three levels of spatial organization can contribute to positive autocorrelation; namely the neighbourhood of (a) ramets, (b) clone fragments and (c) entire clones. Here we use data from one of the most exhaustive sampling schemes for any clonal plant to date to measure the contribution of the neighbourhoods of each distinct clonal structure to total spatial autocorrelation. Four plots (256 grid points each) within dense meadows of the clonal marine plant *Zostera marina* (eelgrass) were sampled for genet structure with 9 microsatellite markers (≈ 80 alleles). We found significant coancestry f_{ij}, for all three levels of spatial organization, even when not allowing for joins between samples of identical genets. In addition, absolute values of f_{ij} and the maximum distance with significant positive f_{ij} decreased with the progressive exclusion of joins between identical genotypes. We thus note that the neighbourhood of a clonal plant consists of three levels of organization, which are reflected in different kinship structures. Each of these kinship structures may affect the level of biparental inbreeding and the physical distance of flowering shoots to their outcrossing neighbourhood. The results of this study also emphasize the notion that spatial autocorrelation crucially depends on the scale and intensity of sampling.

Introduction

Spatially limited dispersal in many plant species has led to the prediction that plant populations show kinship structure on a local scale (Levin & Kerster, 1971; Bradshaw, 1972; Heywood, 1991). At the same time, plant interactions, be it competition or facilitation, are usually limited to the immediate neighbourhood of a target individual (Harper, 1977). Hence, kinship structuring in the immediate neighbourhood will lead to interactions, such as competition for outcrossing opportunities (Price & Waser, 1979), between close relatives. This pattern is likely to influence the evolutionary dynamics of a population (Maynard Smith, 1976; Nakamura, 1980). If our interest in population dynamics arises from an evolutionary context, then it is important to quantify the population genetic structure at this local scale.

Genetic structuring in natural populations can be visualized and explored by the powerful tool of spatial autocorrelation (Sokal & Oden, 1978a). In general, spatial autocorrelation can be defined as the property of variables taking values, at pairs of locations a certain distance apart, that are more similar (positive autocorrelation) or less similar (negative autocorrelation) than expected for randomly associated pairs of observations (Legendre, 1993). Under limited dispersal of seeds and/or pollen, nearest neighbours should provide the highest autocorrelation because kinship is expected to diminish monotonically with distance (Kimura & Weiss, 1964).

Spatial autocorrelation of genotypic data has been investigated mainly in non-clonal plant species (Sokal & Oden, 1978a; Legendre, 1993; Smouse & Peakall, 1999; reviewed in Heywood, 1991). In contrast, clonal plants which often dominate aquatic and terrestrial vegetation (Groenendael & Kroon, 1990; Klimes et al., 1997) need special attention. The complex growth geometry in this large group of successful plant species is prohibiting a straightforward use of spatial autocorrelation. Clonal growth leads to the spatial clustering of modules from one genetic individual. The physical connection between modules often breaks over time through rotting of their rhizomes and/or disturbance events, leading to three distinct levels of spatial organization of clones, namely: (a) The effectively physiologically independent unit (sensu Watson & Casper, 1984; Callaghan et al., 1992) (≈ ramet) which are organized in (b) clone fragments, separated by intruding growth of dominant genets and/or disturbance, and finally (c) the entire clone as the sum of all physiologically independent units belonging to one distinct multilocus genotype. Due to the spatial disruption of clones, the genetic individuals can often only be reconstructed after sampling and subsequent identification with genetic markers (Suzuki et al., 1999).

Most studies, using autocorrelation of genotypic data in clonal plants have focused on

quantifying the degree of aggregation for units of single clones within the sampled areas (clonal structure (a) from above) (Caujapecastells & Pedrolamonfort, 1997; Caujape-Castells et al., 1999; Major & Odor, 1999; Suzuki et al., 1999; Burke et al., 2000). However, including all possible joins between samples in spatial autocorrelation of a clonal plant without taking the spatial spread of the genetic individuals into account, will have the effect that significant autocorrelation cannot distinguish between limited gene flow and clonal growth. We are aware of only two autocorrelation studies, which have explicitly considered the fact that individual clones can cover larger areas. In a study on allozyme polymorphism in a stand of *Quercus chrysolepsis*, Montalvo et al. (1997) found that compared to an analysis including all possible joins (clonal structure (a) from above), significant positive autocorrelation of coancestry over the shortest distance class (2 m) decreased after excluding joins between clonemates (clonal structure (c) from above). Because absolute values were still substantially higher than expected for full and half-sib progeny, they assumed that a large amount of the genetic structure uncovered over short distances is in fact due to clonal growth and might have been caused by scoring errors and/or somatic mutations. In another study on the clonal plant *Zostera marina* (eelgrass), Reusch et al. (1998) used standard deviate autocorrelation of microsatellite allele frequencies to compare a full analysis, including all samples (clonal structure (a) from above), with an analysis on a reduced data set, excluding joins within genets (clonal structure (c) from above). They found no significant autocorrelation over short distances when considering the spatial spread of genets and concluded, that clonal growth alone was responsible for the positive autocorrelation on the full data set. The size of the sampled area (20-m x 80-m) was chosen according to estimates by Ruckelshaus (1996) for neighbourhood areas of c. 20 – 25 m (525 m^2). For *Zostera marina*, direct measurements of seed movement (Orth et al., 1994) and seed and pollen movement (Ruckelshaus, 1996) have shown that dispersal distances are no more than a few meters. Short dispersal distances of propagules seem to be widespread among species of the marine coastal habitat despite the usually strong water movement (Denny & Shibata, 1989; Engel et al., 1999). In the light of these empirical studies, the lack of positive autocorrelation over short distances in *Zostera marina* is unexpected. If the neighbourhood area was smaller in the Baltic than the 20 – 25 m estimated by Ruckelshaus (1996) for Pacific populations, then the scale of sampling in the study of Reusch et al. (1998) might have been too coarse to capture kinship structure. It is known that patterns of spatial genetic autocorrelation are scale dependent and therefore influenced by the chosen sampling scheme (Epperson, 1993). For example, the spatial scale of sampling should be smaller than the spatial autocorrelation (Sokal & Oden, 1978b; Epperson & Li, 1997). In addition each distance class should contain an adequate number of pairwise

comparison (N > 60) to give reliable autocorrelation estimates (Doligez & Joly, 1997; Epperson & Li, 1997). The study of Reusch et al. (1998) intended to cover 4 neighbourhood areas based on the limited information available at that time. However, if dispersal distances were even more limited than assumed then the statistical power to detect kinship structure among nearest neighbours may have been too low (20-m x 80-m plot, 80 random samples) because only few joins fell within the smallest distance classes analysed.

In the study on *Quercus chrysolepsis*, Montalvo et al. (1997) report 5 cases in which two clones separated by > 4 m within a plot share a common genotype. It is unlikely that the resolution of the chosen markers was not high enough to separate these common genotypes into different clones, because overall the probability for chance encounters was low (< 0.01). Alternatively, the 5 cases reported may represent spatially independent clusters of one clone (structure (b) from above). Including these identical multilocus genotypes as different clones in the analysis, will inflate autocorrelation estimates and may have been responsible for the very high coancestry values observed in their study.

The aim of the present study was to use genotypic data from an extensive fine scale sampling in monospecific meadows of the clonal plant *Zostera marina* along the Baltic Coast. Our goal was (i) to evaluate the contributions of the three levels of clonal structure to spatial autocorrelation and hence, to local kinship structure and (ii) to test whether kinship structure in the local neighbourhood of clones is higher than expected from random dispersal. We expected a decrease in the absolute values of coancestry and in the maximum distance with significant spatial genetic structure with the progressive exclusion of joins between like genotypes. In addition, from the empirical evidence of limited dispersal in this species, we expected to find kinship structure beyond the spatial spread of clones. Sampling was extensive both spatially (1-m intervals, 256 grid points, 4 plots) and genotypically (9 microsatellite markers representing $\approx$ 80 alleles). We used regularly spaced lattices of sampling points for their desirable properties in an autocorrelation context (Epperson, 1993). The lattices were square plots to minimize edge effects and the plots were replicated both within and between two populations to verify that the spatial scale of sampling was within the context of a larger spatial pattern (Epperson, 1993).

Materials and methods

Study species, area and sampling

Zostera marina (eelgrass) is a marine angiosperm with a true subaqueous mating system (Den Hartog, 1970). Along the non tidal Baltic Coast the species is perennial and forms dense meadows through a mixture of sexual reproduction and clonal growth. In each of two Baltic *Zostera marina* populations we sampled two 15-m x 15-m plots on a regular grid in 1-m intervals using SCUBA. The 'Falkenstein' population grows on the west side of the Kiel Förde, Schleswig Holstein (54°24' N, 10°12' E) in 3 - 3.5 m water depth. The 'Maasholm' population grows in an estuary at the mouth of the river Schlei, Schleswig Holstein (54°41' N, 10°00' E) in 1.5 - 2.5 m water depth. The 'Maasholm' population grows in an embayment and depending on the speed and direction of the wind the water level can vary up to 60 cm (Marahrens, 1995). The shallower waters of Maasholm make this population prone to disturbance by swan grazing and ice scour.

Leaf material (3 - 5 cm) of the shoots closest to the grid points was collected and preserved through drying in silica-gel for DNA amplification. Total genomic DNA was extracted with the Qiagen plant DNA extraction kit (Qiagen, Hilden, Germany). The DNA-extract was processed in two combined PCR-amplifications using 9 polymorphic microsatellite markers specifically designed for *Zostera marina* (representing ≈ 80 alleles). PCR-reactions with fluorescently labeled primers followed standard protocols (Reusch et al., 2000). Alleles were scored on ABI 377 and ABI 3100 automated sequencers, using the software packages GeneScan 3.1 or 3.7 and Genotyper 2.0 or 3.7 (Biosystems, 1998; 2001). Intercalibration samples assured consistent allele designation across both sequencers. This produced 256 pixel images of the genet composition in the plots (Fig. IV.1). All typed samples of one distinct genotype in each plot were treated as a clone. No clone occurred in more than one plot and the likelihood that ramets were erroneously assigned to the same genet because they exhibited the same nine-locus genotype by chance was very small (all $P_{gen} << 0.001$) (Parks & Werth, 1993).

Autocorrelation analysis with multilocus genotypes

We performed spatial autocorrelation on the genotypic plot data from the two populations to quantify the genetic structure and to assess the effect of a decomposition of clonal structure

on autocorrelation estimates. For the pairwise comparison we generated three different data sets ((a) – (b) – (c)) using MATLAB 6.12. codes (Math Works, 2000). Each data set corresponds to one aspect of the clonal structure as illustrated in Fig. IV.2.

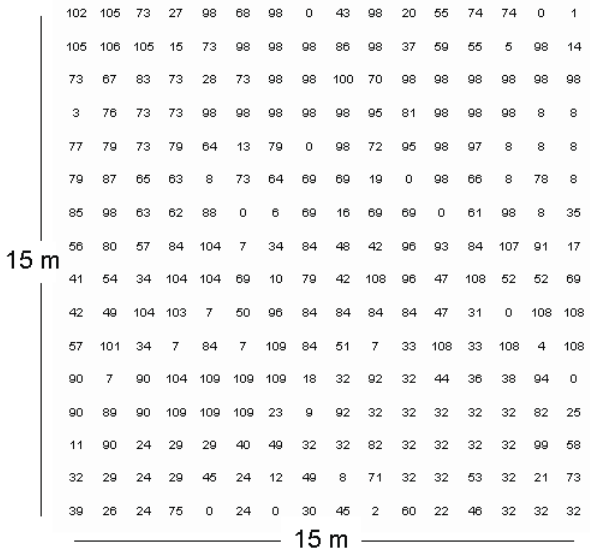

Fig. IV.1: Sampling grid of a 15-m x 15-m plot in a continuous meadow of *Zostera marina* (eelgrass) in c. 3 m water depth off the Baltic Coast. Gridpoints are indicated by running numbers for each distinct multilocus genotype detected through genotyping leaf samples from each grid point with 9 polymorphic microsatellite markers.

(a) Including all samples, the number of included samples represents the size of a given genotype.

(b) Including only one sample from each fragment with a rooks definition of neighbourhood. Other neighbourhood definitions (e.g. queens definition) yield similar results. The coordinates were moved to the centre point of each fragment. The centre points were calculated as $S(xy) = \frac{\sum_{ij}(x,y)_{ij}}{n_i}$ where x,y are the grid coordinates of the *j* sample of clone *i* and *n* is the total number of samples of clone *i*.

(c) Including only the centre point of the largest fragment. If ≤ 2 large fragments had the same area, one of the largest fragments was chosen at random. Here we present only the analysis with the largest fragments. Other choices of fragments such as the smallest or a random fragment yielded similar results.

Genetic correlations between individuals can be summarized over a range of distance intervals in terms of a multilocus estimate of coancestry (Cockerham, 1969) or kinship (Barbujani, 1987). The advantage of genetic structure statistics such as the kinship coefficient is that they have a well-developed foundation in population genetic theory and provide a natural means of combining data over alleles at a locus and over loci (Heywood, 1991).

Following the methods of Loiselle et al. (1996) and Burke et al. (2000), we estimated the coancestry (f_{ij}) of all possible pairs of individuals within each population from their multilocus genotypes; f_{ij} measures the correlation in the frequencies of homologous alleles at each locus for each pair of mapped ramets (Cockerham, 1969) as $f_{ij} = \frac{\sum_{ij}(p_i - \bar{p})(p_j - \bar{p})}{kp(1-\bar{p})} + \frac{1}{2(N-1)}$ ($i < j$), where p_i and p_j are the frequencies of homologous alleles of *i*, *j* pairs of mapped individuals, with the population sample allelefrequency $\bar{p}$ and *k*, the total number of possible pairwise

connections located a discrete number of map units apart and *N* is the total number of samples used in each analysis. Mean values of f_{ij} were obtained for discrete distance intervals (1 m in all four plots, minimal distance 2 m, maximal distance 16 m) by averaging over all pairs of sampling points located within that interval as $\sum p_i(1-p_i)$. To obtain a multilocus measure of spatial genetic structure, the results were combined over loci by weighting each locus by its expected heterozygosity (He).

To assess statistical significance,f_{ij} values were compared to 95% confidence envelopes generated under the null hypothesis of no spatial genetic structure. Specifically, included samples (depending on the analysed clone structure (a), (b), (c)) were drawn at random with replacement and assigned to occupied map locations within each population. This procedure war repeated 399 times, with the observed f_{ij} representing the 400th statistic in each distance class. For a given distance class, f_{ij} is significantly different from zero at $P < 0.05$ if the observed value falls above or below 95% of these statistics. When $f_{ij} = 0$, there is no significant correlation among individuals at the spatial scale of interest; when $f_{ij} > 0$, individuals in a given distance class are more closely related than expected by chance; and when $f_{ij} < 0$, individuals within a given distance class are less related than expected by chance. It has been suggested that each distance class should contain at least 30, preferably > 60 pairwise comparison for reliable estimates (Doligez & Joly, 1997; Epperson & Li, 1997). In the present work, each distance class contained > 160 pairwise comparisons (mean = 772 ± 44), and hence estimates seem to be sufficiently reliable. Calculations were performed using programs developed by J. Nason (University of Iowa). For f_{ij} we used FijAnal version 2.1 and for the bootstrap estimates BS_fij version 2.1. Both programs can be downloaded from the site http://www.nceas.ucsb.edu/papers/geneflow/software/index.htm.

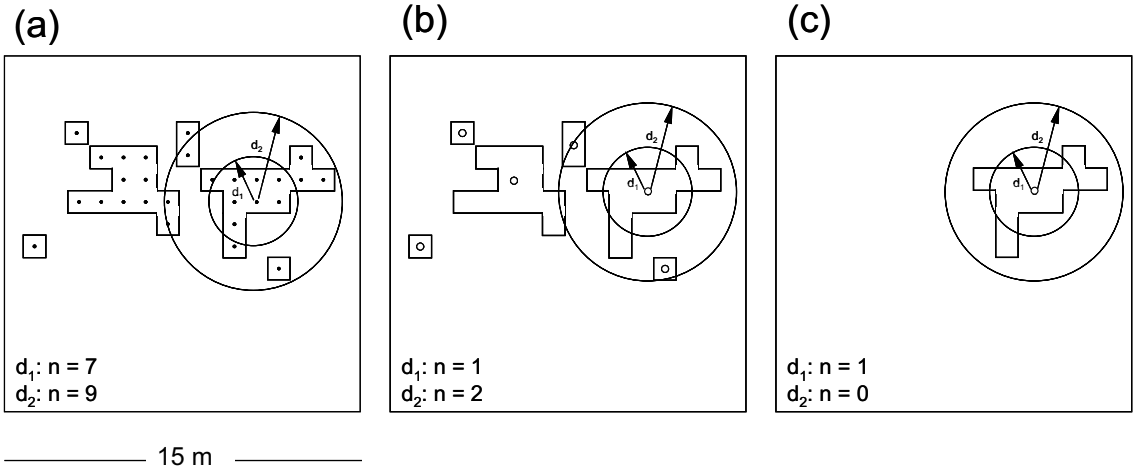

Fig. IV.2: Definition of joins among genotypes in *Zostera marina* (eelgrass) representing three different subsets of genotypic data that were analysed with spatial autocorrelation using multilocus microsatellite genotypes; (a) Including all samples, (b) centre points of all clone fragments and (c) centre point of the largest clone fragment. The two circles in each plot represent two distance classes (d_1 and d_2) and demonstrate the progressive exclusion of joins between like genotypes (a – c) by a decrease in the number of samples (n) with identical multilocus genotype included in each distance class.

Results

The definition of joins as generated by the three different data sets that were analysed with autocorrelation on multilocus genotypes are presented in Fig. IV.2. Each of these data sets represents the genetic neighbourhood of one specific level of organization of a clonal plant. The neighbourhood of a single unit (≈ ramet) is most closely represented by the full data set including all original sampling points (Fig. IV.2a). The neighbourhood of clone fragments (spatially independent aggregations of units) is represented by a reduced data set with only one sample per fragment and the coordinates moved to the centre. Finally, the neighbourhood of the genetic individual is represented by the data set that includes only one sampling point (e.g. centre point of the largest fragment) per genet.

All autocorrelation values were significantly higher than expected from a random pattern over short distances both for the Falkenstein and Maasholm plots (Fig. IV.3). As expect-

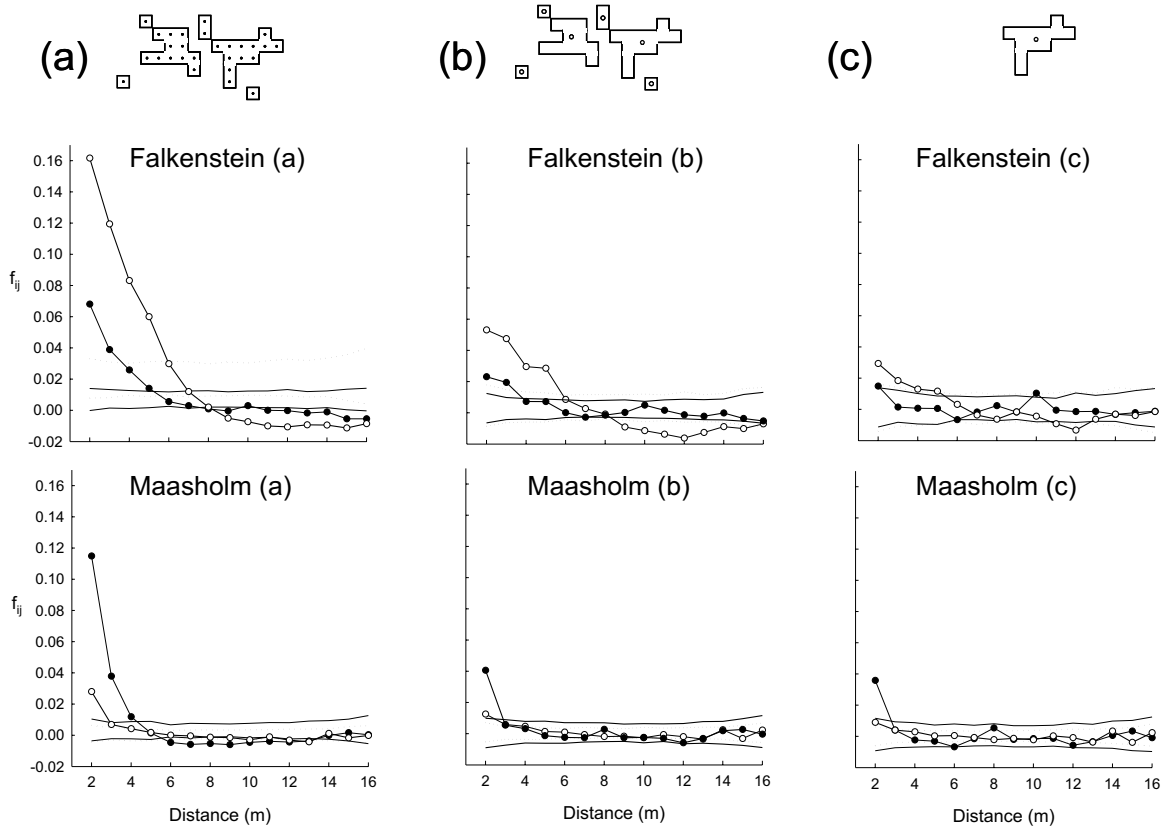

Fig. IV.3: *Zostera marina* (eelgrass) autocorrelation of coancestry (f_{ij}) performed on genotypic data (a) including all samples, (b) including center points of all fragments and (c) including center point of largest fragment for two plots (plot 1 open circles; plot 2 closed circles) in each of two populations Falkenstein and Maasholm. Lines give 95% confidence envelopes around the null hypothesis $f_{ij} = 0$ for plot 1 (solid) and plot 2 (dotted) in each population. Note that the scale of all axes is indicated only along the periphery, for reasons of clarity.

ed, the absolute values for f_{ij} were highest when all samples were included in the analysis (Fig. IV.3a). Here the aggregation of units with identical genotype through clonal expansion leads to high average f_{ij} values. The values decreased sharply with the stepwise decomposition of the clonal structure, excluding first joins within clone fragments, and then between any identical clone members (left to right Fig. IV.3 (a) – (c)). Within the shortest distance class (2 m), all f_{ij} were significantly positively correlated, but their absolute values varied between replicated plots both within and among populations (Table IV.1). It has been proposed that the x-intercept can be considered a measure of the spatial scale of autocorrelation (Upton & Fingleton, 1985; Epperson & Li, 1997). Likewise, the intercept with the confidence envelopes should provide a conservative estimate of the spatial scale with significant autocorrelation. The intercept of positive autocorrelation with the upper confidence limit decreased from 5 m in Falkenstein and 4 m in Maasholm to 2 m in both sites (Table IV.1) with the progressive exclusion of joins between identical genotypes. While for a single unit, the neighbourhood of up to 6 m radius consisted of close relatives (Fig. IV.3a), for fragments (Fig. IV.3b) and whole genets (Fig. IV.3c) only the immediate neighbourhood was close kin. The plot pairs from each population showed similar values for the maximum distance of significant f_{ij} , but varied greatly in absolute values of f_{ij} (Fig. IV.3 and Table IV.1).

Table IV.1: Spatial autocorrelation of genotypic data in two Baltic Sea *Zostera marina* populations. Absolute f_{ij} values for the shortest 2 m distance interval (f_{ij}s), maximum distance in meters with significant positive autocorrelation (MD) for three data sets each representing one aspect of the clonal structure: (a) physiologically independent units (all samples), (b) spatially independent aggregations of units (all fragments of each genet) and (c) the genetic individuals (one sample per genet). N_{genets} indicates the number of multilocus genotypes found in each plot, based on 9 polymorphic microsatellite markers.

	Falkenstein				Maasholm			
	Plot 1		Plot 2		Plot 1		Plot 2	
Data set	MD	f_{ij}s	MD	f_{ij}s	MD	f_{ij}s	MD	f_{ij}s
(a)	5	0.068	5	0.161	4	0.114	3	0.028
(b)	3	0.023	5	0.053	2	0.04	3	0.013
(c)	2	0.015	4	0.028	2	0.036	2	0.009
N_{genets}		109		101		122		201

Discussion

Based on one of the most exhaustive spatial sampling of genotypes in a plant population thus far, we have demonstrated that local genetic patterns are non random at different levels of clonal structure in two populations of *Zostera marina* on the Baltic Coast. The significant positive autocorrelation for the data set representing only one individual of each clone (Fig. IV.2c)

revealed kinship structure beyond the spatial aggregation of units (≈ ramets) belonging to identical genotypes, suggesting limited gene flow in *Zostera marina* meadows.

The small scale kinship structuring detected in this study emphasises the fact that spatial autocorrelation crucially depends on the scale of sampling (representation of dispersal distances) and in the case of clonal plants, on the sampling scheme (representation of clonal structure). The sampling points lay within 1-m intervals. On average, independent rhizome units are c. 10 cm in length and $1m^2$ is estimated to contain about 60 - 70 such units (A. Hämmerli, unpublished data). This means that the analysis including all samples could underestimate the density of effectively physiologically independent units by a factor of 70. Exhaustive sampling of all units is hardly possible for areas of 256 m^2 and therefore the 1-m sampling grid must serve as a proxy for the level of independent ramets (Fig. IV.2a). The neighbourhood definition for sampling points with identical multilocus genotype influences the size and number of clone fragments (Fig. IV.2b). For example, a queens definition of neighbourhood (grid points with identical multilocus genotype sharing only one corner belong to the same clone fragment) would reduce the number of fragments in Fig. IV.2b from 6 to 4. On average this would not affect the clone structure (Fig. IV.2c). More important than the exact number and size of clone fragments is to recognize that clones are fragmented not only at the level of physiologically independent ramets but also at the higher organizational level of ramet clusters. The spatially separated groups of trees with identical genotype also indicated such a structure to be present in *Quercus chrysolepsis* (Montalvo et al., 1997). In the latter study, it was probably responsible for values of coancestry too high to be explained solely by limited dispersal. Spatial autocorrelation representing the clones (Fig. IV.2c) requires the choice of only one sample per genotype, which most simply could be defined as the centre point of a clone. However, in the case of several clone fragments (Fig. IV.2b) more than one centre point can be defined. In this study we chose the centre point of the largest fragment because this fragment is expected to lay on average most closely to the origin of initial recruitment of a given clone. Analysis with the centre point of the smallest or a random fragment of each clone only slightly affected absolute values and changed the results only little.

In the absence of inbreeding the expected value of f_{ij} for full and half-sib progeny is 0.25 and 0.125, respectively (Queller & Goodnight, 1989). If we assume linear generations without backcrossing and low levels of inbreeding (as is known for *Zostera marina* (Ruckelshaus, 1995)), then $f_{ij} \approx (0.5)^n$ with n = number of generations. This yields an estimated maximum number of generations necessary to reach the observed significant f_{ij} values for the clone neighbourhood (Fig. IV.3c, Table IV.1) of between 4 and 6 generations. This is most probably an overestimate of the generations needed to establish such a neighbour-

hood because *Zostera marina* is self-compatible (de Cock, 1980) and backcrosses can be expected. Exact values are speculations, but the conservative rough estimates indicate that the immediate neighbourhood of a target clone is surprisingly young. In the context of other studies on coancestry it seems however, that absolute values can vary greatly among species. Our values from the analysis including all samples range from 0.028 to 0.161 for the 2 m interval (Table IV.1). For the same distance, autocorrelation values from Louisiana iris hybrid populations showed a range of ≈ 0 – 0.2 (Burke et al., 2000) and for populations of canyon life oak ≈ 0.38 – 0.42 and 0.25 excluding multiple samples per genet. For the marine environment J. Coyer has reported significant coancestry values of 0.0601 – 0.0926 for *Zostera noltii* (Jim Coyer, personal communication) at the 2 m scale and J. Olsen reported significant coancestry of 0.25 for *Ascophyllum sp*. on a scale of 40 cm – 1 m (Jeanine Olsen, unpublished data). Both studies correspond to kinship among unique genotypes with no confounding clonal structure and their values are relatively high compared to the range of 0.009 – 0.015, excluding joins within genets, from this study (Table IV.1).

Dispersal distances for seed and pollen in *Zostera marina* were found to be no more than a few meters (Orth et al., 1994; Ruckelshaus, 1996). In the sampled plots we found significant kinship structure only within 2 – 4 m from a target individual, which seems to be in line with the empirical data. However, these figures could underestimate the neighbourhood area for eelgrass because the size of the panmictic breeding unit may be influenced by several factors. (i) We took the confidence limit intercept as threshold for both the absolute values of f_{ij} and the maximum distance with significant kinship structure. For the maximum distance, this threshold may be too conservative (Upton & Fingleton, 1985; Epperson & Li, 1997). Taking the x-intercept as a threshold instead would yield distances of 6 – 10 m. On the other hand, values within confidence limits should be interpreted with caution because they lay within the boundaries of random variation. Using standard normal deviate for spatial autocorrelation of 5 allozyme loci in *Zostera marina*, Ruckelshaus (1998) used the x-intercept and estimated patch sizes of between 80-m x 80-m and 64-m x 64-m. This seems very large compared to the values from this study. (ii) To explore the clone level, the coordinates of the original sampling points were moved to the centre, which slightly reduced the physical distance to neighbouring points in the pairwise comparison. (iii) All clones within each of the 256 m^2 plots were found to be in Hardy-Weinberg equilibrium which indicates that the scale of panmixis may be larger than estimated from the maximum distance with significant coancestry.

There has been much debate on occasional dispersal of both seed and pollen through rafting shoots (Ruckelshaus, 1998; Reusch, 2002). Although most observations of this process are anecdotal evidence it is well possible, that rafting of flowering shoots is con-

tributing to the local gene pool, influencing the size of the genetic neighbourhood through long-range dispersal. Even when taking the above-mentioned points into consideration, an assumed neighbourhood size of 525 m^2 (Ruckelshaus, 1996) seems to overestimate the size of the panmictic breeding unit at least for the observed Baltic Sea populations of this study. For a more thorough understanding of both short- and long-range dispersal it would be desirable to be able to distinguish between the contributions of pollen and seeds to the local genetic structure. This may be possible in the near future with the establishment of maternal markers.

Another question is whether genet density within local populations reflects the initial colonising cohort and its subsequent survivorship (initial seedling recruitment) or a continuous recruitment of genets (repeated seedling recruitment) (Eriksson, 1993). Still very little is known about recruitment patterns in *Zostera marina* populations, and seagrass species in general. Recruitment through seedlings in the dense meadow is considered a rare event (Robertson & Mann, 1984; Olesen & Sand-Jensen, 1994b; and personal observation). Although quantitative data are lacking for our study site, it is well possible that seedling recruitment is still abundant with respect to the potential life span of clones (Reusch et al., 1999). In general, for long-lived organisms such as clonal plants, assuming restricted dispersal, populations will have exceedingly long memories of initial colonization patterns and subsequent disturbance events (Heywood, 1991). If seedling recruitment is extremely low (low turnover) then this “memorizing effect” should reduce kinship structuring and possibly cancel out significant autocorrelation signals. Because we find significant positive autocorrelation over short distances we would argue that, although rare, continuous seedling recruitment is contributing to the local gene pool.

One of the consequences of clonal growth is increasing between-ramet selfing (geitonogamy) with increasing distance of flowering shoots from their outcrossing neighbourhood. Our data show that as a direct consequence of kinship clusters, outcrossing between related individuals (biparental inbreeding) may augment the effects of geitonogamy. Both, geitonogamy (Handel, 1985; Eckert, 2000; Reusch, 2001) and biparental inbreeding (Price & Waser, 1979; Levin, 1989; Fenster, 1991; Waser & Price, 1994) have been shown to result in reduced offspring fitness. Hence for pollen of a single flowering shoot within a clone, the mating landscape with increasing distance to its outcrossing neighbourhood is a continuum of between-ramet selfing, biparental inbreeding and full outcrossing. The decomposition of the clonal structure (Fig. IV.3, left-right) can be viewed as a simulation of reduced physical distance to the full outcrossing neighbourhood with an increase in the chance for successful fertilization and fitter offspring. If this effect translates into selection pressure for outcrossing in

the natural environment, then the tendency for spatial disintegration of larger clones into fragment clusters may be adaptive in *Zostera marina*. This could be an interesting starting point for future experiments.

In the present study we have shown that the stepwise decomposition of clonal structure reduces the positive spatial autocorrelation both in absolute terms and in terms of the maximum distance with significant deviation from random expectations. Even with this reduction, considering the spatial spread of genetic individuals, clones grow in a neighbourhood of significant kinship structure. It will be desirable for future studies to have direct measurements of recruitment patterns in *Zostera marina* meadows to get deeper insight into the connection between pattern and process.

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Chapter - V

Drying equipment after sampling in Maasholm

Isolated eelgrass shoots - outposts of new recruitment? - remnants of a dying clone? - or the “tree line” of an eelgrass meadow?

Genet demography of a marine clonal plant based on marker assisted clone identification

Summary

Clonal plants are often fragmented, which makes it difficult to identify their genetic individuals on a scale large enough to follow them over time, without the use of polymorphic genetic markers. This is the reason why in the past, most empirical work on clonal plant species in the natural environment has either studied shoot demography or genet composition rather than genet demography. The aim of the present study was (i) to establish a general method for the study of genet demography and (ii) to use this method to track clones of the marine flowering plant *Zostera marina* between the years 2000 and 2001 in two plots (256 m^2 each) on the Baltic coast. The goal was to obtain dynamic estimates of growth, mortality and recruitment at the level of the genetic individuals. In addition, we counted the number of flowering and vegetative shoots to get a measure of reproductive output at the level of single genets. In contrast to the high population stability found for *Zostera marina* based on shoot demography (Olesen & Sand-Jensen 1994b), we found high variability within the two sampled plots in terms of genet turnover, growth and reproductive output. While this variability was very pronounced across genets, patterns were almost constant within genets through time. This suggests a genetic component to the balance between flowering intensity and the production of vegetative shoots. In addition, larger clones showed higher growth rates and a higher increase in reproductive output, which is in line with previous findings that large clones of *Zostera marina* were more heterozygous than small ones. The study method is discussed in the light of possible difficulties revealed in the two years of sampling.

Introduction

Seagrasses are the most productive macro organisms of coastal waters worldwide. They have great importance in providing habitat and resources for a diversity of animal species (Orth & Moore 1983a), filtering seawater and stabilizing the sediment (Tomlinson 1974). Seagrasses have come into focus of research in the past decades for two main reasons: (i) The large-scale decline that was observed in many areas (denHartog 1996; Orth & Moore 1983b; Rasmussen 1973; Short & Wyllie-Escheverria 1996) has promoted studies on restoration (Lent & Verschuure 1995; Orth et al. 1999; Pranovi et al. 2000; Van Katwijk et al. 1998; Williams & Davis 1996; Worm & Reusch 2000; reviewed in Fonseca et al. 1988) and genetic diversity (Procaccini & Mazzella 1998; Reusch et al. 1999; Ruckelshaus 1998; Waycott 1998). (ii) In relatively undisturbed areas seagrasses form closed monospecific meadows through clonal growth. In a system with only one plant species, interspecific interactions can be neglected and hence, these meadows have been recognized as an excellent study system for the biology of clonal plants and in particular their breeding system (Reusch 2000; Reusch 2001; Ruckelshaus 1995; Ruckelshaus 1996).

Clonal plants are unique in their ability to expand their genetic individuals in space through reiteration of rhizome modules. As a consequence, clones have a potentially infinite life span, which makes life history hard to track. This may be the reason why theoretical work on reproductive effort (Armstrong 1982; Gardner & Mangel 1999), clonal integration (Oborny et al. 2000), fitness components (Winkler & Fischer 1999) and senescence (Gardner Shea & Mangel 1997) has usually addressed the level of the genetic individual (the clone) while in contrast most empirical work has either investigated shoot demography (e.g. Donohue et al. 2000; Ishii & Takeda 1997; Nantel & Gagnon 1999) or has described genet composition (e.g. Ellstrand & Roose 1987; Kreher et al. 2000; Persson & Gustavsson 2001; Stehlik & Holderegger 2000). Genet demography, the direct measurement of recruitment, mortality, growth and reproductive effort at the level of single clones is not well studied (Eriksson 1993). This is especially true for seagrasses where genet demography would not only help to better understand the evolutionary dynamics of the system but in addition could provide key information on the persistence and suitability of selected clones for restoration purposes.

Eelgrass (*Zostera marina* L.), the dominant seagrass species of the northern hemisphere is probably the most thoroughly studied seagrass species in terms of shoot demography (Olesen & Sand-Jensen 1994a; Olesen & Sand-Jensen 1994b; Olesen & Sand-Jensen 1994c) and genet composition (Reusch et al. 1998; Reusch et al. 2000a; Ruckelshaus 1998; Williams 2001). The general consent is that eelgrass meadows are highly productive

systems with some unknown factors stabilizing genet diversity at relatively high levels. The dynamics of single shoots and the description of genet structure are increasingly well known. However, only the direct measurement of genet dynamics can merge population level shoot dynamics and genet structure into a foundation for the study of evolutionary questions. There have been attempts to reconstruct genet demography from their estimated size. For example (Reusch et al. 1998) assumed a yearly centrifugal spread of 13 cm from a hypothetical founder plant in the centre of the genet and estimated the age of clones of c. 10 m^2 to be between 30 – 70 years old. However, the radial spread of 13 cm had to be measured as growth increments into open space or adjacent mussel (*Mytilus edulis*) beds (Reusch et al. 1994) while estimates for clone sizes came from the closed meadow. Hence, such estimates would clearly benefit from a method that could directly measure genet growth in closed stands.

The aim of the present study was (i) to establish a general method for the study of genet demography in closed monospecific stands of clonal plants such as seagrasses and (ii) to use this method for estimates on genet demography in a Baltic Sea population of eelgrass (*Zostera marina*). To this end we used high-resolution genetic mapping in two plots within a closed Baltic Sea eelgrass meadow. With microsatellite markers we tracked clones in the years 2000 and 2001 to get dynamic estimates of growth, mortality and recruitment at the level of the genetic individuals. In addition, we counted the number of flowering and vegetative shoots to measure reproductive output and density at the level of single clones in the two years of measurements.

Materials and methods

Species and study area

Eelgrass (*Zostera marina* L.) is the dominant seagrass species of the northern temperate zone forming dense meadows through clonal growth. Its leaf canopy and root structure has significant importance as habitat for invertebrate and fish and for the stabilisation of the coastal sediment (Den Hartog 1970). In the non-tidal Baltic Sea eelgrass is perennial and occupies space mainly through the clonal expansion of rhizomes. Additionally, plants reproduce sexually with an unknown contribution of sexual to vegetative reproduction. Flowering shoots are produced once a year and can be easily distinguished from vegetative shoots in

an early stage of development. Flowering shoots start to grow in winter and early spring, while flowering and seed ripening takes place during the summer months (de Cock 1980; Den Hartog 1970).

Sampling took place in Falkenstein on the west side of the Kiel Förde, Schleswig Holstein (54°24' N, 10°12' E) in 3 - 3.5 m water depth. The shallow waters of Falkenstein are home to a large relatively undisturbed continuous meadow of *Zostera marina*.

Mapping of genets with microsatellite markers

To study the demography of clones, high resolution genet mapping was conducted in Falkenstein in two consecutive years. During the month May and June 2000, two 15-m x 15-m permanent plots were set up (F_1 and F_2) using SCUBA. The plots were located within c. 100 m of each other. Both plots were permanently marked with PVC posts in 1-m intervals at the base and the top line. With the aid of a measuring tape attached to these posts, each grid-line was marked in 1-m intervals with tagged flexible posts. The plots were sampled on a 1-m regular grid (256 points). Leaf material (3 - 5 cm) of the plants closest to the grid points was collected and preserved through drying in silica-gel for DNA amplification. If no plant was present within a radius of 10 cm around the grid point the position was counted as non-vegetated. Total genomic DNA was extracted with the Qiagen plant DNA extraction kit (Qiagen, Hilden, Germany). The DNA-extract was processed in a combined PCR-amplification with 24 cycles using the 4 most polymorphic microsatellite markers (GenBank accession no. AJ249307, AJ249305, AJ009900, AJ009898) specifically designed for *Zostera marina*. PCR-reactions with fluorescently labeled primers followed standard protocols (Biosystems 1998). Alleles were scored on an ABI 377 automated sequencer, using the software package GeneScan 3.1 and Genotyper 2.0 (Biosystems 2001). This produced 256 pixel images of the genet composition in the plots (Fig. V.1). The same two plots were resampled after one year in 2001. Alleles were scored on a new ABI 3100 automated sequencer with the software packages GeneScan 3.7 and Genotyper 3.7 (Reusch et al. 2000b). Intercalibration samples assured consistent allele designation across both sequencers. A total of 998 samples were genotyped for all 4 loci. Very few samples were not successfully genotyped (F_1 = 12; F_2 = 5) and these were excluded from the analysis. The remaining empty pixels were grid points from which no leaf samples could be collected and thus corresponded to non-vegetated space at the time of sampling. The relatively low number of empty pixels (F_1 = 1; F_2 = 8) indicates that the plots were positioned within a larger area of the closed meadow. The likelihood that ramets were erroneously assigned to the same genet because they exhibited the same four-locus

genotype by chance was very small (all P_{gen} << 0.001) (Parks & Werth 1993) and therefore all typed samples of one distinct genotype in each plot were treated as a clone. No genotype occurred in more than one plot.

Mapping of density and flowering

In addition to the genetic mapping we counted the number of vegetative and flowering shoots in 40-cm x 40-cm sub-quadrates around each grid-point between June - July 2000, and 2001 to obtain an estimate of shoot density and reproductive output m^{-2} $genet^{-1}$ for the sampled areas. In *Z. marina* flowering shoots are produced once a year and they can be distinguished from vegetative shoots in an early stage of development. Data from an eelgrass population in Denmark (c. 100 km from our site) showed that the appearance of new flowering shoots levels off in June and that the turnover is low during the summer month (Olesen 1999). This could be confirmed with a random subset of 10 sub-quadrates in each plot that were re-measured 1 month after the first census in 2000. No significant change in flowering intensity could

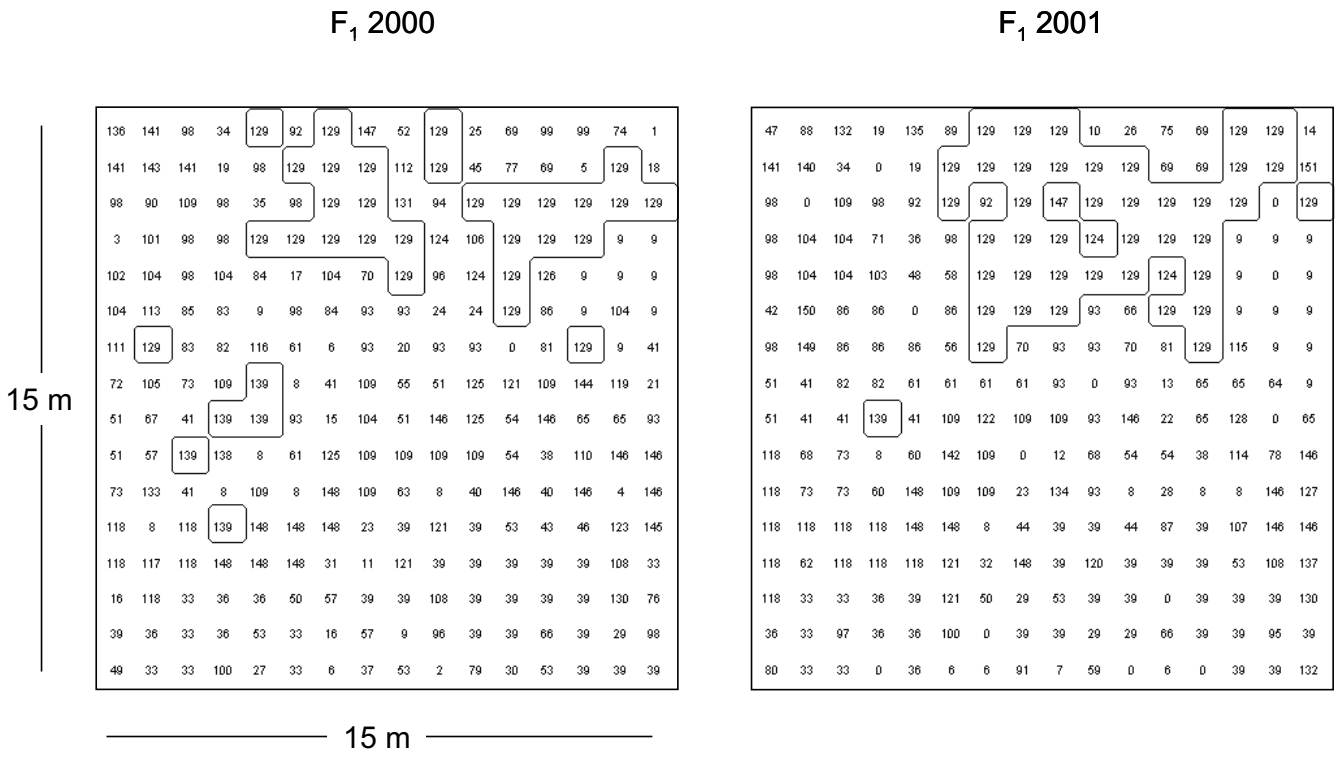

Fig. V.1: Change in area for a 15-m x 15-m plot within a dense eelgrass (*Zostera marina*) meadow in Falkenstein (German Baltic Coast) between the years 2000 and 2001. Grid points are indicated with running number of each distinct multilocus genotype. Identical genotypes detected in the two years of sampling have identical numbers. Each grid point corresponds to 1m^2 of meadow according to our definition. One whole plots corresponds to a 256 m^2 pixel image of the genet structure with a resolution of 1m. Two clones are given as examples for an increase and a decrease in area. Clone nr. 129 increased in area from 29 to 40 m^2, and clone nr. 139 decreased from 6 to 1 m^2.

be observed during this period (paired t; all $p > 0.13$). We thus assume that a single census is indicative of reproductive investment during one entire season.

Response variables and statistical analysis

In each plot genet size was estimated as the number of samples with identical multilocus genotype x m^2. For the same grid points we calculated the mean density of vegetative and flowering shoots. The values obtained from 40-cm x 40-cm quadrates were divided by 0.16 to get comparable estimates m^{-2}. Flowering % was calculated as (number of vegetative shoots) / (number of flowering shoots) counted in each 40-cm x 40-cm quadrate. Growth was calculated as (clone size in 2001) – (clone size in 2000) and consequently, relative growth was calculated as [(clone size in 2001) – (clone size in 2000)] / (clone size in 2000). Paired t-tests were performed at the clone level to test for differences in the mean values of clone size, and the number of vegetative and flowering shoots between the two years of sampling. Relative growth could not be tested in this way because defining a relative size of clones besides relative growth did not seem sensible. Genet recruitment (r_g) was calculated as $r_g = (n_r/ n_0)*100$ and genet mortality (m_g) was calculated as $m_g = (n_d/n_0)*100$ with n_r as the number of recruits, n_d the number of dead (Table V.2) and n_0, the total number of genotypes detected in 2000 (Table V.1). Because leaf samples were collected within a 10 cm radius around the grid points, measurements could deviate up to 40 cm in the two consecutive years. Any measurement error resulting from such deviation is expected to be random and hence to go into the residual error. However, it is possible that it affected genet diversity in the smallest size class of clones ($1m^2$) because our sampling resolution was not higher than 1 m^2. This was also indicated in a breakpoint in the frequency distribution of the number of genets recorded as dead in each size-class. The smallest size-class (1 $m^{2)}$ accounted for 86 % of clones recorded as dead in 2001. Therefore recruitment and mortality estimates were compared to recalculated values excluding this smallest size class. The two plots were close replicates in terms of the measured traits and data was therefore pooled for correlations. For data preparation (e.g. clone assignment and clone matching between the two years) we used MATLAB 6.12. codes (Math Works 2000) and for the statistical analysis GENSTAT 5 (Payne 1997).

Results

Phenology at the plot level

Means over genets for the measured variables in each plot and year are summarized in Table V.1. Values for genetic diversity (P_D), Density m^{-2} and flowering m^{-2} were slightly higher for plot F_1 in both years. The number of genets detected in 2001 was consistently lower than in 2000. This holds even in the unlikely case that each untyped sample would have added one distinct genet. There was also a consistent increase in both the mean number of flowering shoots and flowering %. Overall the two plots were close replicates in terms of the measured variables.

Table V.1: Phenology of two 15-m x 15-m plots within a dense Baltic Sea eelgrass meadow for two years of measurement 2000 and 2001. Given are the number of distinct multilocus genotypes (N_{genets}), the number of samples typed for the four most polymorphic microsatellite markers, empty grid points, the number of samples untyped for some loci, genotypic diversity ($P_D = N_{genets} / N_{samples}$ (4 typed) (Ellstrand & Roose 1987), number of vegetative and flowering shoots m^{-2} and the percentage flowering ± 1 SE of the mean. Note that the sample size for standard errors corresponds to N_{genets} .

	F_1 2000	F_1 2001	F_2 2000	F_2 2001
N_{genets}	104	89	94	76
$N_{samples}$ (4 typed)	255	244	253	246
Empty	1	0	3	5
$N_{samples}$ (< 4 typed)	0	12	0	5
P_D	0.408	0.365	0.372	0.309
Vegetative shoots m^{-2}	347 ± 19.9	390 ± 49.2	337 ± 53.2	321 ± 65.3
Flowering shoots m^{-2}	31 ± 5.07	59 ± 24.6	16 ± 2.27	38 ± 7.46
Flowering %	4.9 ± 0.50	7.6 ± 0.85	4.4 ± 0.50	11.1 ± 2.19

Dynamics at the plot level

The number of genets detected in both years of sampling (survivors), the number of new genets detected in 2001 (recruits) and the number of lost genets in 2001 (dead) is summarized in Table V.2. Genet mortality (m_g) and recruitment (r_g) was surprisingly high even if corrected for possible measuring errors in the smallest size class of 1 m^2. For both, corrected and uncorrected values, the number of lost genets was consistently higher than the number of recruited genets for both plots. The sampled areas were within the closed meadow and hence any increase in clone area and or density must be closely linked to a decrease in area and density of neighbouring clones. Therefore it was more sensible to partition the

Table V.2: Dynamics of two 15-m x 15-m plots within a dense Baltic Sea eelgrass meadow for two years of measurement 2000 and 2001. Given are the number of distinct multilocus genotypes detected in both years (Survivors), the number of genotypes only detected in 2000 (Dead) and the number of genotypes only detected in 2001 (Recruits). From these values genet recruitment (r_g) and genet mortality (m_g) were calculated for the whole data set and corrected for measurement errors in the smallest size class (1m^2) (see text for details).

	F_1	F_2
Survivors	42	42
Dead	62	52
Recruits	47	34
r_g (%)	45.2	36.2
m_g (%)	59.5	55.2
r_g corrected (%)	11.43	10
m_g corrected (%)	25	21.87

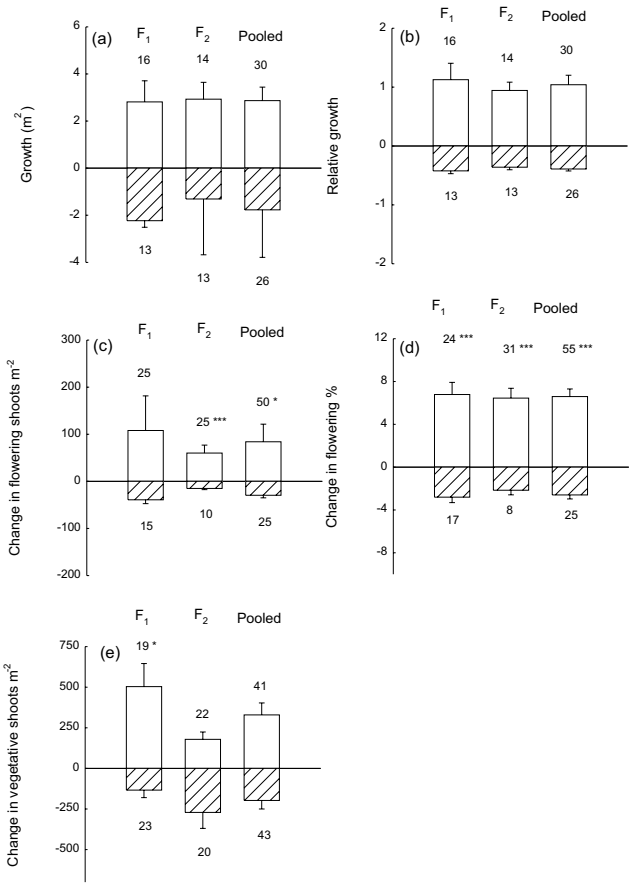

Fig. V.2: Dynamics for growth (a), relative growth (b), flowering shoots (c), flowering % (d) and vegetative shoots (e) for two plots (F_1 and F_2) and for the pooled data, within a Baltic Sea eelgrass meadow. The dynamics of each variable is indicated as positive (empty bars) or negative (hashed bars) change ± 1SE of the mean. The number of genets for each mean value is given above and below each error bar. Significance levels of paired t-tests between the mean values of each year of measurement are indicated as * 10%, ** 5% and *** 1%. Note that relative growth was not tested (see text for details).

change into increasing and decreasing values with 0 as the threshold. The mean positive and negative change for the measured variables is presented in Fig. V.2. There was a significant increase in the number of flowering shoots for plot F_2 and for the pooled data (Fig. V.2c). Both plots showed a significant increase in flowering % (Fig. V.2d). Plot F_1 showed a slight increase in the number of vegetative shoots over the two census years (Fig. V.2e).

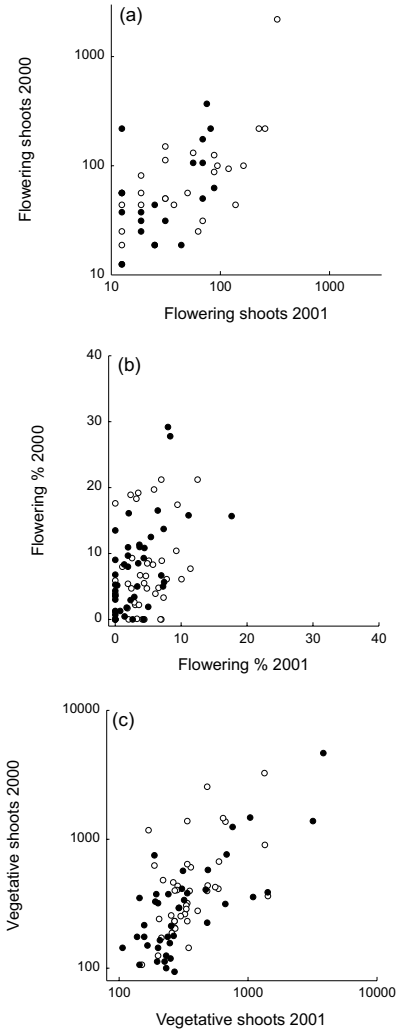

Fig. V.3: Eelgrass (*Zostera marina*). Temporal correlations of the number of flowering shoots (a), flowering % (b) and the number of vegetative shoots (c) for measurements in 2000 and 2001 at the level of the genetic individuals (clones). The data was pooled for the two sampled 15-m x 15-m plots, Falkenstein 1 (open circles) and Falkenstein 2 (filled circles). Note that the scale of both axis in (a) and (b) is logarithmic.

Dynamics at the genet level

The measured variables flowering shoots, flowering % and vegetative shoots at the level of the genetic individuals are presented in Fig. V.3. All correlations were significant with ($r = 0.65$; $F_{1,82} = 62.03$; $p < 0.001$) for flowering shoots m^{-2}, ($r = 0.41$; $F_{1,82} = 18$; $p < 0.001$) for flowering % and ($r = 0.70$; $F_{1,82} = 70.85$; $p < 0.001$) for vegetative shoots m-2. Clones with a high number of flowering shoots, a high flowering intensity and a high number of vegetative shoots in 2000, showed on average high values in these traits one year later.

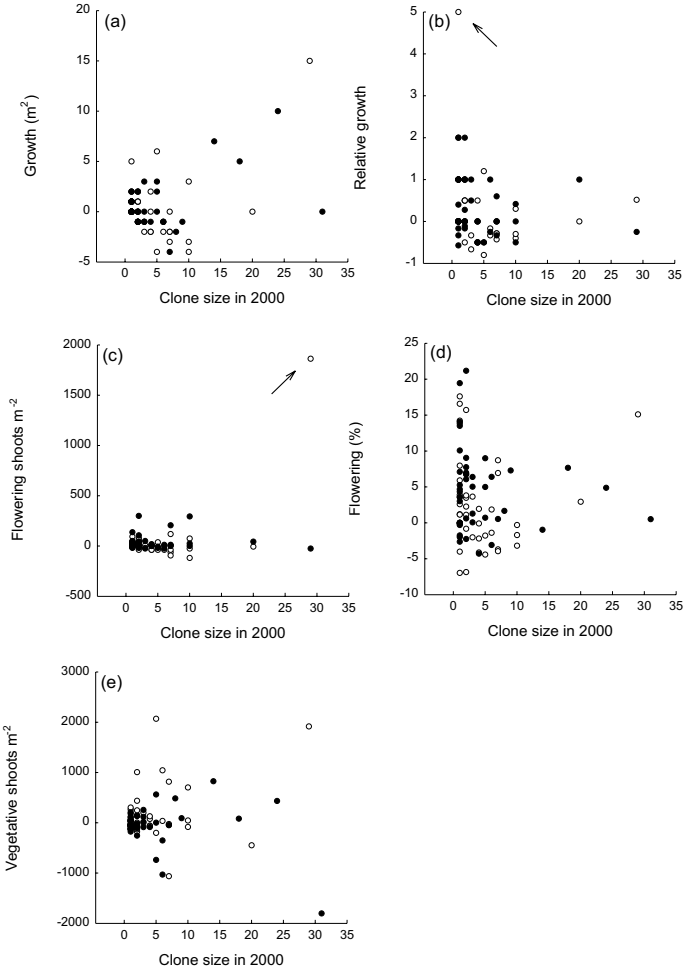

Fig. V.4: Eelgrass (*Zostera marina*). Correlations of growth (a), relative growth (b), number of flowering shoots (c), flowering % (d) and number of vegetative shoots (e) against clone size in the first year of sampling (2000). Data was pooled for the two sampled plots Falkenstein 1 (open circles) and Falkenstein 2 (filled circles). Arrows in (b) and (c) indicate outliers (see text for details).

Dynamics for size-classes

Change in the measured variables against the size of each clone in 2000 is presented in Fig. V.4. Absolute growth was significantly positively correlated with the size of clones in 2000 ($r = 0.40$; $F_{1,82} = 17.49$; $p < 0.001$) (Fig. V.4a) but this was not the case for relative growth ($r = 0.07$; $F_{1,82} = 1.47$; $p = 0.228$) (Fig. V.4b). The change in number of flowering shoots was also significantly positively correlated with clone size in 2000 ($r = 0.53$; $F_{1,82} = 34.40$; $p < 0.001$) (Fig. V.4c) but not for flowering % ($r = 0.0$; $F_{1,82} = 0.05$; $p = 0.820$) (Fig. V.4d) and vegetative shoots ($r = 0.0$; $F_{1,82} = 0.19$; $p = 0.662$) (Fig. V.4e). Correlations of relative growth and flowering shoots with clone size in 2000 each showed one outlier value indicated with an arrow in Fig. V.4b,c. Correlations

were recalculated without these outliers to assure that significance or non-significance was not caused by these single values. Without the outliers correlations were ($r = 0.04$; $F_{1,81} = 1.19$; $p = 0.278$) for relative growth and ($r = 0.37$; $F_{1,81} = 14.34$; $p < 0.001$) for flowering shoots respectively and hence the outliers did not affect significance.

Discussion

Eelgrass has shown high population stability in terms of shoot density between successive years (Olesen & Sand-Jensen 1994b) similar to other perennial clonal herbs of contrasting growth patterns (Callaghan 1976; Doust 1981; Hutchings 1979; Noble et al. 1979). Genet level demography in this study has shown high variability within only one year, consistent for two 256 m^2 plots in a dense eelgrass meadow. In dense stands, demography of individual shoots (emerging shoots, ramets etc.) can be expected to be relatively stable because any eventual gap that will occur through shoot mortality will be filled through new recruitment. Such stability however masks any dynamics that might be present at the level of single clones because an increase in area of one clone will require a decrease in area of another clone in order to keep the meadow closed (Fig. V.1).

The sampling scheme that was chosen for the present study has some desirable properties but also associated difficulties. Sampling regular grids rather than random point within a given area allows the establishment of permanent plots and the production of pixel images that can be repeated through time. A static grid efficiently maps the dynamics of spatial patterns such as clone structure but is restricted by the chosen resolution (Epperson 1993). We chose a resolution of 1-m because according to genet size estimates from a random sampling (Reusch et al. 1998) such a minimum distance would yield a representative sample of the genet diversity and at the same time, sampling and replication of relatively large areas (256 m^2) would still be feasible. Because of the very skewed size distribution of genets (median at 1 m^2) it is possible that the number of genets detected in both years underestimates the actual number of especially small genets present in the sampling areas. In addition, there is some variability of sampling around each grid point, which could contribute to a bias in the number of small clones. The plots were chosen as squares to reduce edge effects. Despite this precaution, it is clear that in several cases the area of clones at the edges may have been underestimated because they extended beyond the boundaries. In future studies it will be desirable to measure genet diversity at smaller scales for a subsample of quadrates to estimate the bias in the number of small clones that comes from the chosen mesh size. In

the same study site, (Reusch et al. 1998) for example found 2 and 7 clones in two square meter areas for which all rhizomes were excavated and genotyped.

The density of flowering and vegetative shoots and flowering % seem to be a relatively stable property of clones. While we found for example a significant increase in flowering % after one year (Fig. V.2d), flowering percentage plotted for single clones and both years was still highly correlated (Fig. V.3b). It was not observed that clones with very low flowering % in 2000 changed to very high values of flowering % in 2001 and vice versa. Strong correlations were also present for the density of flowering and vegetative shoots consistent for both plots. This may indicate a genetic component to the measured traits. It is unlikely that environmental heterogeneity is responsible for the strong correlations because such heterogeneity would have to closely follow the spatial patterns of single clones. In addition the abiotic environment within the meadow seems relatively homogeneous over the sampled distances. It is also unlikely that a physiological constraint for flowering or the production of vegetative shoots (e.g. threshold in resource accumulation) in rhizome fragments would be visible at the level of single clones. In many clonal plant species, large rhizome fragments flower more often and produce more vegetative offspring than small fragments (Cain 1990; Cain & Damman 1997; Carlsson & Callaghan 1990; Eriksson 1988; Steven 1989). Similarly it was shown that the age of rhizome fragments influenced the biomass production of vegetative shoots in eelgrass (Olesen & Sand-Jensen 1994b). However, single clones, depending on their size, are expected to contain cohorts of several 100 to 1000 such fragments of different size and age (Reusch et al. 1998 and Hämmerli unpubl. data). In addition, mean density of flowering and vegetative shoots and flowering frequency was not significantly correlated with clone size (data not shown), which supports the expectation that such physiological constraints would be averaged out at the genet level.

Assuming a constant radial spread of clone margins across genotypes, the areal growth will increase with increasing size of the clone. For example taking the growth increment from (Reusch et al. 1994) as 13 cm and two clones of 2 m^2 and 4 m^2 initial size respectively, then their increase in area will be 1.02 m^2 and 1.41 m^2 respectively. Such a relationship may be reflected in the positive correlation between growth and initial clone size (Fig. V.4a). It is interesting to note that for small clones a 13 cm growth increment corresponds well to the areal growth found in this study. Yet, still larger clones can show considerably higher growth values (Fig. V.4a). It is not yet clear, however, why large clones showed a higher increase in mean density of flowering shoots over a one year period than small clones, as indicated by the significant positive correlation between the change in the number of flowering shoots m^{-2} and initial clone size (Fig. V.4c). Two measurement points in time are not

enough to extrapolate any trend. For example the significant increase in flowering intensity over the measured time period could be part of a cycle driven by climatic factors, a chance event or could be well within the long term variability of this particular eelgrass population. The finding that on average larger clones were able to perform greater changes in the measured traits is, however, supporting the finding that clone size is indicative of genet fitness. In a study on inbreeding depression, (chapter II this thesis) we found that larger clones on average were more heterozygous, which may explain the higher lateral expansion. In the present study there was no indication that small clones could somehow compensate the putative advantages (larger growth increment, stronger increase in flowering) of large clones. This brings up the question of what keeps small clones (e.g. rare genotypes) from going extinct and what stabilizes genetic diversity at the observed levels. It seems likely that chance events like disturbance (ice scour in very cold winters) open the meadow for more abundant seedling recruitment and set the stage for a deterministic process, namely competitive exclusion. This has to be investigated further.

In conclusion, the observed genet dynamics of the two sampled plots within a dense eelgrass meadow showed an overall high variability across clones. Patterns were relatively constant for single clones indicating a genetic component to flowering intensity and the production of vegetative shoots. In addition, the change in clone size and flowering intensity was indicating an advantage for large clones over smaller clones for the measurement period. Permanent plots sampled on regular grids provide a valuable starting point for the study of genet level dynamics, but remeasurements over several years will be needed to evaluate the high turnover rates found in this study on a broader scale.

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Conclusion

CONCLUSION

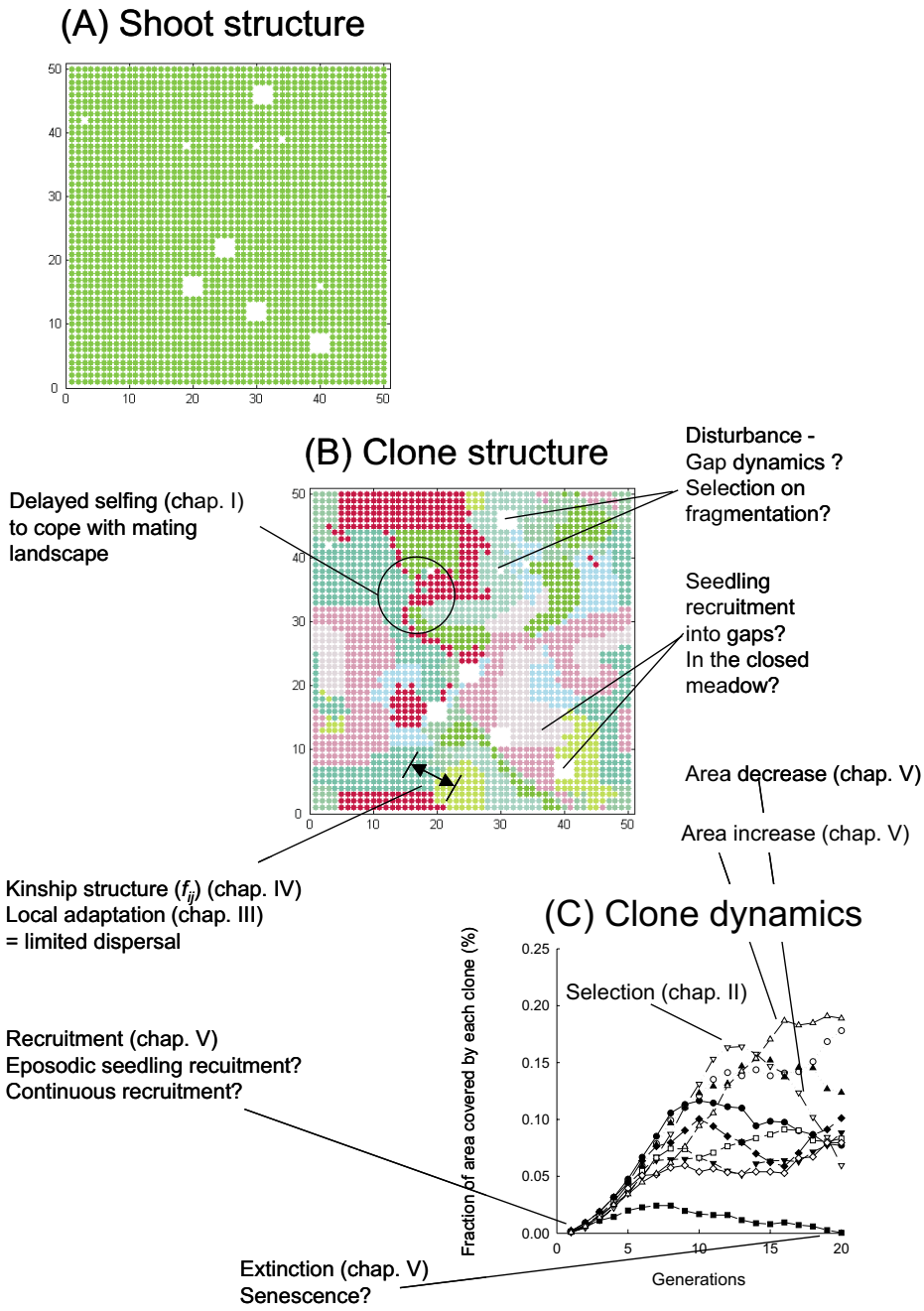
When swimming over the Falkenstein eelgrass meadow for the first time, I got the impression of seamless homogeneity. What I saw was a vast forest of luscious green that lost itself in the turbidity of the Baltic. In contrast, the picture that emerges from the various aspects of clone structure discussed in the previous five chapters reveals an utterly different, much more mosaic and dynamic view. The thorough uncovering of the clonal structure peeled off the layer of homogeneity and revised the impression I got when I first saw an eelgrass meadow.

Each chapter has its own share in dismantling this picture of homogeneity. But, putting the pieces together, what are the general insights into the processes moulding the genetic structure in eelgrass meadows and where are still large gaps in knowledge that wait to be explored? Two factors seem to profoundly influence the genet structure and the mating landscape of eelgrass clones. Selection for outbred clones (chapter II) and limited seed and pollen dispersal, which are in turn indicated by kinship structure (chapter IV) and local adaptation (chapter III). If delayed selfing by means of a cryptic self incompatibility system is adaptive (chapter I), then eelgrass individuals seem to be well equipped to cope with an environment in which the availability of cross pollen is highly variable and its concentration low compared to self pollen. And finally, genets recruit, die and change their size, observable even within the short time period of one year (chapter V). This indicates that eelgrass clones do not settle in a genetic neighbourhood that is stable over time, but rather move through a shifting patchwork of clones and clone fragments.

This evolutionary view into eelgrass genetics and the interactions between clones does also have implications in a conservation restoration context. The measurement of single eelgrass shoots would have never revealed the patchiness and dynamics that is present at the clone level. Yet only this patchiness tells us that there are large individuals and small individuals and that some individuals perform better than others. This is why addressing the clone level is also relevant for population persistence, and ultimately, for ecosystem functioning. A major concern in eelgrass conservation is genetic erosion. Could the depletion of eelgrass individuals lead to the collapse of a population? From the results presented in the previous chapters this seems well possible. The loss of eelgrass clones will ultimately lead to an increasing proportion of inbred individuals. If the outbred individuals vanish, there will soon be nothing to select for but the bare sand (chapter II). In addition, there is no more use for delayed selfing if the chance of receiving cross pollen is zero (chapter I). These negative effects will even be amplified, because the degree and also the spatial extent of kinship structure (chapter IV) will increase in response to a loss of heterozygotes. In a genetically depau-

perate meadow, long range dispersal through rafting shoots as a source for new genetic input is most probably insufficient, because the major contribution seems to come from short dispersal (chapters III and IV). If unfavourable conditions persist long enough, eelgrass coverage will finally decrease. This may make recolonization through transplantation of eelgrass rhizomes or even seeds unavoidable. But, does it matter which plants we take and if we have several populations to choose from which one is better suited? Can marker data assist us in these decisions? In the case of eelgrass we run into a paradox situation because genetic markers are necessary to find out whether marker information is conclusive (chapter III). In principal markers should not be used as sole information for the selection of transplants. Marker variability alone does not reveal local adaptation and hence cannot avoid the risk of outbreeding depression in the performance of transplants. Yet, markers are necessary to identify the clones in the first place and allow the selection of replicated transplants to test at the phenotypic level, whether local adaptation occurs.

Clearly there are still large gaps in knowledge about the factors influencing the genetic structure of eelgrass meadows. The direct measurement of seedling recruitment is one such gap, the effects of disturbance events is another (see also next Figure). *Terra incognita* is still abundant on the map of eelgrass population structure, keeping up curiosity in this fascinating green underwater landscape.



This picture is a synthesis of the findings from chapters I - V. Illustration of the shoot structure (A) gives the impression of great homogeneity while the corresponding clone structure (B), reveals a patchy landscape. Each pixel in (A) and (B) represents one shoot or ramet. Each colour in (B) represents a unique genotype. The lattices correspond approximately to a 5-m x 5-m square of eelgrass meadow. Imagine that the patch image in (B) is the result of 20 generations of ramet production (corresponds to ca. 1 – 2 vegetation periods) of 10 eelgrass clones competing for space*. Then the dynamics of these 10 clones over the 20 generations might look similar to what is presented in (C). Selection, increase and decrease in area, extinction and recruitment all con tribute to the outcome in (B). The numbers in brackets correspond to the chapters in this thesis. Questionmarks indicate still largely unknown aspects of the clone structure in eelgrass meadows.

*The corresponding MATLAB code is a cellular automaton written and developed by the author. It is designed as an explorative tool to investigate genet dynamics by simulating different recruitment events, allocation patterns and levels of disturbance at a chosen spatial scale. The codes and a detailed description of the state transitions can be found in Appendix2. The program can be downloaded from http://www.mpil-ploen.mpg.de/english/physeco/staff/hammerli.htm . The code can easily be extended for different scenarios.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GLOSSARY: Often used definitions and synonyms

Alleles	Different forms of a gene found at the same locus, assumed to arise by mutation
Anther	Male organ in a plant, releasing pollen
Apomixis	Reproduction without fertilization
Clone	Propagating population of genetically identical organisms
DNA	Deoxyribonucleic acid, the genetic material of all cells and many viruses
DNA polymerase	Enzymes involved in template-directed synthesis of DNA from deoxyribonucleotide triphosphates
Gene	Set of DNA sequences that are required to produce a single polypeptide; "Unit of inheritance"
Genet (see clone)	
Genetic drift	Random change in allele frequency within a population
Genetic erosion	Negative effects of a gradual loss of genetic diversity within a population
Genetic locus	The position of a gene on a chromosome
Hermaphrodite	Both sexes in the same individual
Heterosis	Hybrid vigour, the superiority of a heterozygotic organism over the homozygote
Heterozygote	Nucleus, cell or organism with different alleles of one or more specific genes
Homozygote	Nucleus, cell or organism with identical alleles of one or more specific genes
Inbreeding	Mating between closely related individuals
Inbreeding depression	Negative effects of inbreeding
Kinship structure	The degree of relatedness among neighbouring individuals
Markers	Identifiable physical locations on a chromosome
Maternal effects	Phenotypic traits that are determined by the genotype of the mother through maternal gene products in the egg.
Microsatellite	Short sequences of di- or trinucleotide repeats of very variable length. Using PCR primers to the unique sequences upstream and downstream of a microsatellite their location and polymorphism can be determined.
Monoeceous	Male and female reproductive organs in the same individual
Mutation	Change in DNA sequence of an organism
Outcrossing	Mating between relatively unrelated individuals
PCR	Polymerase chain reaction. The first practical step for *in vitro* amplification of DNA.
Primers	Short preexisting polynucleotide chain to which new deoxyribonucleotides can be added by DNA polymerase
Ramet	Independent member of a clone that is potentially physiologically independent
Rhizome	The root of a clonal plant
Rhizome unit	Closely corresponds to ramet, but is the actually independent member of a clone
Selfing	Self-fertilization (opposite to outcrossing)
Spadix	The fleshy axis of a spike
Stigma	Female organ in a plant, receiving pollen (see also anther)

APPENDIX 1

Example of imput text file with column description* for the startup and scissors MATLAB programs. Only the first lines are presented. Such files were processed for clone matching and genet mapping in the sampling plots (see chapter 3 of general introduction).

y1	x1	flp1	sh1	fl1	h1	Ga2a	Ga2b	Ga17Ha	Ga17Hb	Ga23a	Ga23b	Ga35a	Ga35b	tag
0	0	0.09756098	41	4	58	118	118	121	141	169	169	96	104	152
0	1	0.10526316	38	4	66	116	118	129	129	169	171	98	100	153
0	2	0.07894737	38	3	71	116	118	129	129	169	171	98	100	151
0	3	0.07317073	41	3	68	118	118	129	139	169	171	98	100	148
0	4	0.03703704	54	2	57	116	118	127	129	167	169	98	98	155
0	5	0.04918033	61	3	52	116	118	129	129	169	171	98	100	147

flp2	sh2	fl2	Ga2a2	Ga2b2	Ga17Ha2	Ga17Hb2	Ga23a2	Ga23b2	Ga35a2	Ga35b2	taG2
40	1	0.025	118	118	129	129	169	169	94	96	257
50	2	0.04	116	118	129	129	169	171	98	100	258
50	0	0	116	118	129	129	169	171	98	100	259
61	2	0.033	0	0	0	0	0	0	0	0	260
52	1	0.019	116	118	129	135	169	169	96	96	261
46	1	0.022	114	114	125	137	169	171	118	130	262

y; x: x,y coordinates in sampling plot

flp: flowering %

sh: number of vegetative shoots

fl: number of flowering shoots

h: mid height of canopy at sampling location

Ga#: allele number for the four most polymorphic microsatellite markers; a) smaller, b) larger allele

tag / taG: unique number for each collected sample

1, 2: year of measurement (1=2000; 2=2002)

* Column headings have to be omitted in order to load file into MATLAB

```
%STARTUP PROGRAM FALKENSTEIN PLOT 1

%load falkenstein plot data (four most polymorphic markers)

load  d:\zostera\data\falkrem\falk1rem.txt

%assign variable to each column
x1=falk1rem(:,2);
y1=falk1rem(:,1);
flp1=falk1rem(:,3);
sh1=falk1rem(:,4);
fl1=falk1rem(:,5);
h1=falk1rem(:,6);
GA2a=falk1rem(:,7);
GA2b=falk1rem(:,8);
GA17Ha=falk1rem(:,9);
GA17Hb=falk1rem(:,10);
GA23a=falk1rem(:,11);
GA23b=falk1rem(:,12);
GA35a=falk1rem(:,13);
GA35b=falk1rem(:,14);
tag=falk1rem(:,15);

%remeasurement 2001
flp2=falk1rem(:,18);
sh2=falk1rem(:,16);
fl2=falk1rem(:,17);
GA2a2=falk1rem(:,19);
GA2b2=falk1rem(:,20);
GA17Ha2=falk1rem(:,21);
GA17Hb2=falk1rem(:,22);
GA23a2=falk1rem(:,23);
GA23b2=falk1rem(:,24);
GA35a2=falk1rem(:,25);
GA35b2=falk1rem(:,26);
taG2=falk1rem(:,27);

%make clones from allele profile
GA2 = [GA2a GA2b;GA2a2 GA2b2];
GA17H = [GA17Ha GA17Hb;GA17Ha2 GA17Hb2];
GA23 = [GA23a GA23b;GA23a2 GA23b2];
GA35 = [GA35a GA35b;GA35a2 GA35b2];
vecta = linspace(0,0,512);
vectb = vecta';
taG = [tag;taG2];

full = [GA2 GA17H GA23 GA35 taG];
in = find (full(:,1)>0&full(:,2)>0&full(:,3)>0&full(:,4)>0&full(:,5)>0&full(:,6)>0&full(:,7)>0&full(:,8)>0&full(:,9)>0);
out = find (full(:,1)==0|full(:,2)==0|full(:,3)==0|full(:,4)==0|full(:,5)==0|full(:,6)==0|full(:,7)==0|full(:,8)==0|full(:,9)==0);
miss = sortrows(taG(out,:))
rmiss = size(miss,1)

innew = full(in,:);
clf = sortrows (innew, [1 2 3 4 5 6 7 8 9]);
clone = [clf(:,1) clf(:,2) clf(:,3) clf(:,4) clf(:,5) clf(:,6) clf(:,7) clf(:,8)];
tag1 = clf(:,9);
r = size(clone,1);
for i=1:512


            trans = clone';
            first = trans(:,1);
            expand = repmat(first,1,r);
            result = trans-expand;

  r1 = find
```

```
(result(1,:)==0&result(2,:)==0&result(3,:)==0&result(4,:)==0&result(5,:)==0&result(6,:)==0&result(7,:)==0&result(8,:)==0);
   r2 = find (result(1,:)~=0|result(2,:)~=0|result(3,:)~=0|result(4,:)~=0|result(5,:)~=0|result(6,:)~=0|result(7,:)~=0|result(8,:)~=0);

   tag3 = tag1(r1,:);
   match = ismember(taG,tag3);
   rmatch = find(match==1);
            clone = clone(r2,:);
   tag1 = tag1(r2,:);

   vectb(rmatch)=i;
   clear tag3;
   [r,c] = size(clone);
   if r==0
      break
   end

end

vectb1 = vectb;

count = 1;
countre = 257;

%produce matrices from vectors

for i=1:16
   for j=1:16
      X1(i,j) = x1(count);
      Y1(i,j) = y1(count);

      Z1(i,j) = flp1(count);
      F1(i,j) = fl1(count);
               D1(i,j) = sh1(count);
               H1(i,j) = h1(count);
      Cl1(i,j) = vectb(count);
      TAG(i,j) = tag(count);
      %remeasurement 2000
      Z2(i,j) = flp2(count);
      F2(i,j) = fl2(count);
               D2(i,j) = sh2(count);
      Cl2(i,j) = vectb(countre);
      TAG2(i,j) = taG2(count);
      VB1(count) = vectb(count);
      VB2(count) = vectb(countre);
      count=count+1;
      countre = countre+1;
   end
end

save matrices for use in scissors.m

save (['X11.txt']),'X1','-ascii');
save (['Y11.txt']),'Y1','-ascii');
save (['Z11.txt']),'Z1','-ascii');
save (['F11.txt']),'F1','-ascii');
save (['D11.txt']),'D1','-ascii');
save (['H11.txt']),'H1','-ascii');
save (['Cl11.txt']),'Cl1','-ascii');
save (['TAG11.txt']),'TAG','-ascii');
save (['Z12.txt']),'Z2','-ascii');
save (['F12.txt']),'F2','-ascii');
save (['D12.txt']),'D2','-ascii');
save (['Cl12.txt']),'Cl2','-ascii');
save (['TAG12.txt']),'TAG2','-ascii');
save (['VB11.txt']),'VB1','-ascii');
save (['VB12.txt']),'VB2','-ascii');
```

```
save (([‘Xn11.txt’]),’x1’,’-ascii’);
save (([‘Yn11.txt’]),’y1’,’-ascii’);

%SCISSORS PROGRAM: calculate variates at the clone/fragment  level

%Choose resolution for contour mapping

res = 18;%resolution for flowering, vegetative shoots etc.
res1 =100;%resolution for clones

cfrag = 1;

%produce sparse matrices for resolution flexibility

mnews = sparse(18,18);
clzero = full(mnews);
Zzero = full(mnews);
Fzero = full(mnews);
Dzero = full(mnews);
Hzero = full(mnews);
clid = sparse(res,res)
CLID = full(clid);
FRAG = full(clid);
CIRC = full(clid);
NFN = full(clid);
MFN = full(clid);
MDN = full(clid);
MHN = full(clid);
NON = full(clid);
NFNC = full(clid);

%choose plot and measurement  year 2000 or 2001 (*F1, *F2), [11 12 21 22]

%load matrices from startup# files

map = 12;
if map == 11;
X1 = load([‘X11.txt’]);
Y1 = load([‘Y11.txt’]);
Z1 = load([‘Z11.txt’]);
F1 = load([‘F11.txt’]);
D1 = load([‘D11.txt’]);
H1 = load([‘H11.txt’]);
Cl1 = load([‘Cl11.txt’]);
TAG = load([‘TAG11.txt’]);
VB = load([‘VB11.txt’]);
vectb = VB’;
x1 = load ([‘Xn11.txt’]);
y1 = load ([‘Yn11.txt’]);
plotnum = 1;
elseif map == 12;
X1 = load([‘X11.txt’]);
Y1 = load([‘Y11.txt’]);
H1 = load([‘H11.txt’]);
Z1 = load([‘Z12.txt’]);
F1 = load([‘F12.txt’]);
D1 = load([‘D12.txt’]);
Cl1 = load([‘Cl12.txt’]);
TAG1 = load([‘TAG12.txt’]);
VB = load([‘VB12.txt’]);
vectb = VB’;
x1 = load ([‘Xn11.txt’]);
y1 = load ([‘Yn11.txt’]);
plotnum = 2;
elseif map == 21;
X1 = load([‘X21.txt’]);
```

```
Y1 = load(['Y21.txt']);
Z1 = load(['Z211.txt']);
F1 = load(['F21.txt']);
D1 = load(['D21.txt']);
H1 = load(['H21.txt']);
Cl1 = load(['Cl21.txt']);
TAG1 = load(['TAG21.txt']);
VB = load(['VB21.txt']);
vectb = VB';
x1 = load (['Xn11.txt']);
y1 = load (['Yn11.txt']);
plotnum = 3;
elseif map == 22;
X1 = load(['X21.txt']);
Y1 = load(['Y21.txt']);
H1 = load(['H21.txt']);
Z1 = load(['Z22.txt']);
F1 = load(['F22.txt']);
D1 = load(['D22.txt']);
Cl1 = load(['Cl22.txt']);
TAG1 = load(['TAG22.txt']);
VB = load(['VB22.txt']);
vectb = VB';
x1 = load (['Xn11.txt']);
y1 = load (['Yn11.txt']);
plotnum = 4;
end
frag = 1;

%choose clones to analyse

m = max(vectb)
start = 1;
finish = m;
%choose file to save data in
plotcode = (['Falkrem',num2str(plotnum),'.txt']);
%plotcode = (['temp',num2str(plotnum),'.txt']);
% area per gridpoint (model = ramets; clone maps = lattice)
scale = 1;

zufall = rand(m,3);
count1 = 1;

%fill sparse matrices with variates and chosen resolution

clzero(2:17,2:17) = Cl1(1:16,1:16);
Zzero(2:17,2:17) = (Z1(1:16,1:16)*100);
Fzero(2:17,2:17) = F1(1:16,1:16);
Dzero(2:17,2:17) = D1(1:16,1:16);
Hzero(2:17,2:17) = H1(1:16,1:16);

Xi = linspace(1,18,18);Yi = linspace(1,18,18);
[Xzero,Yzero] = meshgrid(Xi,Yi);

xi = linspace(1,18,res);
yi = linspace(1,18,res);
[xxi,yyi] = meshgrid(xi,yi);
xi1 = linspace(1,18,res1);
yi1 = linspace(1,18,res1);
[xxi1,yyi1] = meshgrid(xi1,yi1);

ZP = interp2(Xzero,Yzero,Zzero,xxi,yyi,'*cubic');
ZPnew = interp2(Xzero,Yzero,Zzero,xxi1,yyi1,'*cubic');
ZD = interp2(Xzero,Yzero,Dzero,xxi,yyi,'*cubic');
ZH = interp2(Xzero,Yzero,Hzero,xxi,yyi,'*cubic');
colormap white%summer
```

```
pcolor(xxi1,yyi1,ZPnew);shading interp;axis square
hold on
str = num2str((vectb),3);
axis off
text (x1+1.67,y1+2,str,'FontSize',7);
hold on

%loop through clone fragments

for count = 1:m
    count

            cln = (clzero==count);%calc 1 against 0


            zzi = interp2(Xzero,Yzero,cln,xxi1,yyi1,'*nearest');  %cubic (Queens) or nearest (roocks)
   [c,h] = contour (xxi1,yyi1,zzi,[0.5 0.5],'k');axis square;
            hold on;
  if h~[];
  title(['clone ',num2str(count)],'fontsize',7);
  set(gca,'fontsize',7);
  groe = size(h);nobs = groe(1);
  siz1 = size(c);nobs1 = siz1(2);
  c1 = c(2,:);
  c2 = c(1,:);
            pos(1) = 1;
            num(1) = c1(1);

   %from here down, calculations with fragments

  for j=1:nobs;
    Cid(cfrag) = count;
    start(j) = pos(j)+1;stop(j) = pos(j)+num(j);
    graph = c(:,start(j):stop(j));
    xg = graph(1,:);
              yg = graph(2,:);
    %each grid point gets area of contour
    %aa(count1,j) = polyarea(xg,yg);

    inps = (sparse(inpolygon(Xzero,Yzero,xg,yg))>0);
    cords = find(inps);
    [xcord,ycord] = find(inps);
    Zn = Zzero(:);vz = Zn(cords);
    Fn = Fzero(:);vf = Fn(cords);
    Dn = Dzero(:);vd = Dn(cords);
    Hn = Hzero(:);vh = Hn(cords);


    %each grid point gets 1 m^2 * scale
             aa(cfrag) = length(cords)*scale;
     leng = length(cords);

             if leng > 0;
       %look-up matrix for clone ID

    meanz(cfrag) = mean(vz);
    sez(cfrag) = (std(vz)/sqrt(leng));
    meanf(cfrag) = mean(vf);
    sef(cfrag) = (std(vf)/sqrt(leng));
    sumf(cfrag) = (mean(vf/0.16))*leng;
    meand(cfrag) = mean(vd);
    sed(cfrag) = (std(vd)/sqrt(leng));
    sumd(cfrag) = (mean(vd/0.16))*leng;
    meanh(cfrag) = mean(vh);
    seh(cfrag) = (std(vh)/sqrt(leng));
    farbe = zufall(count1,:);
```

```
    %fill(xg,yg,farbe);
    hold on
    frag = frag+1;
    end
                    if j<nobs;
                    k = j+1;
                    pos(k) = num(j)+pos(j)+1;
     num(k) = c1(pos(k));
     end
     cfrag = cfrag+1;
                    end
clear c h zzi
end
count1 = count1+1;
end

%set up table

table = [Cid' aa' meanz' sez' meand' sed' sumd' meanf' sef' sumf' meanh' seh'];

%save table

save (plotcode,'table','-ascii');
```

APPENDIX 2

Description for the cellular automaton zosgrow.m

The program is designed as an explorative tool to investigate genet dynamics in a clonal plant such as eelgrass by choosing the values of various starting conditions and dynamic parameters.

This is the script: Imagine an eelgrass seedling growing in a patch of open sand. The seedling grows to become a clone by extending and branching rhizomes. After some time rhizome connections disintegrate, the clone becomes fragmented and rhizome units (= ramets) compete themselves now for space with other units of the same genetic individual. As the units on the edge of the clones continue growing, central units become more and more disconnected from foreign pollen sources. Finally, the units on the edge will encounter other genotypes and interact according to their own genotype (allocation pattern).

Cellular automaton

I use a cell based approach, where each 'unit' is occupying one cell. Each cell has 8 neighbours that either share a side or a corner, the 'Queens definition' of neighbours. The lattice has torus boundary conditions. Technically the state transitions are set up as a stochastic cellular automaton (see for example Casagrandi & Gatto Nature 400: 560-562);

The simple assumptions are, that units assimilate to allocate into pollen(P), seeds(S) and clonal growth(G). At the start, associations of units are placed on the 'game board'. Each unit is equipped with scores for the three parameters mentioned above and units of the same clone have the same set of scores (the genotype).

State transitions

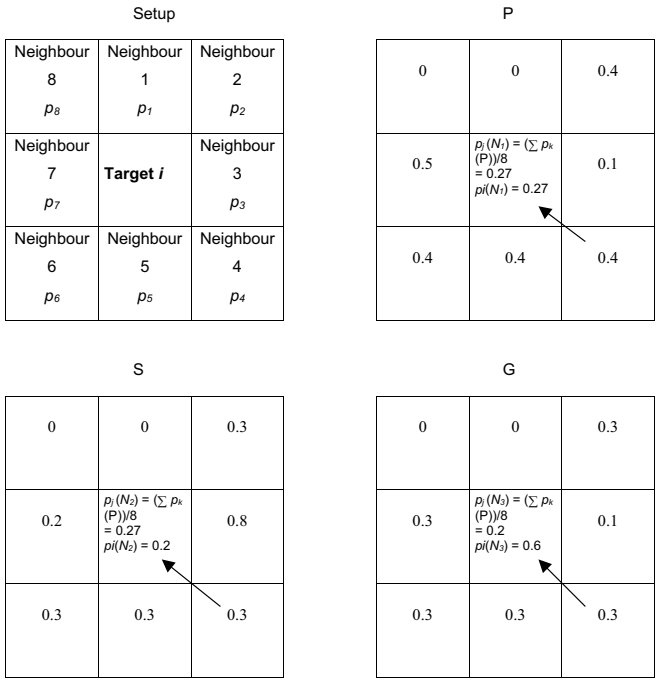
Let N_i for i, j = 1…3 be the function considered, or in other words the pure strategies (S, P, N). With p_i and p_j ($0 \leq p < 1$) being the allocated fractions to each function we get the mixed strategies for the target unit (i) and the neighbourhood (j). Zero belongs to the interval, because a cell can also be empty. We have to keep this in mind, because it is affecting the range of possible outcomes in the scoring, as we shall see later. Then $p_j\,(N_i) = (\sum \text{pk}\,(N_i))/n$ for k = 1…n, and n = 8 in our case, considering eight neighbours. Further more $\sum p_i\,(N_i) = 1$,

or 0 if empty. $\sum p_j (N_i) \leq 1$ because the mean over eight neighbours can include one or several zeros. Each $p_i (N_i) > p_j (N_i)$ is one score for *i*.

If the sum of the scores for *i* is < 2 then one randomly chosen stronger neighbour occupies the site *i* at t+1 (next iteration or time step) or in other words a unit is stronger than the other if it has a higher score in at least two of the three functions. This procedure is illustrated in Fig. 1. If all cells are occupied the possible sum of scores are 1 and 2. As mentioned above, cells can be empty and this has the effect, that 0, 1, 2 and 3 are all possible outcomes.

Clones growing in open space soon adjust to growing squares, which is as close as a matrix representation gets to a circle. This is what we would expect in an environment with low abiotic heterogeneity. As soon as clones touch the edge of another clone, we start to see dynamic patterns. In a homogeneous abiotic environment we expect most asymmetry to occur in places of interaction between species or in single species stands between genets. For a clonal plant such as eelgrass it means that units surrounded by fragments of their own genet are in a 'no win no loose' situation in terms of the competition for space (symmetry of interactions), whereas units on the edge of clones bordering other genets, are in a situation of 'nothing ventured nothing gained' (asymmetry of interactions).

Disturbance is easily introduced by a random opening of gaps within the lattice. The frequency and size of the gaps can be modified.



Example of the state transitions for the model zosgrow.m. The parameter values for the allocation pattern into pollen (P), seeds (S) and clonal growth (G) were chosen arbitrarily. The first matrix shows the set-up for the remaining three matrices. The arrows indicate a possible shift of status for the given configuration. For details see text.

```
%   ZOSGROW
%   explorative cellular automaton to investigate genet dynamics in a clonal plant
%   A. Hämmerli; Plön 2002


%number of iterations (generations of rhizome unit production)
iteration = 20;
%gap size (matrix length)
gapsize = 1;%gapsize*2~gap area
%gap frequency
gapfreq = 5;
%grid resolution (size of lattice)
m = 50;
%number of genets recruited
Nclones = 10;
%genet size
Sclones = 1; %start with one pixel = seedling; few clones and large lattice -
% start with larger genets to save calculation time

gen = (1:iteration)';
matr = sparse(m,m);
C = full(matr);
P = full(matr);
S = full(matr);
G = full(matr);
dot = full(matr);


p = -Sclones:Sclones;
pmax = max(p);
pmin = min(p);

%seeding - initial recruitment of genets
for count=1:Nclones
        kx=floor(rand*(m+(-2-pmax+pmin))+(1-pmin)); ky=floor(rand*(m+(-2-pmax+pmin))+(1-pmin));
        num = rand(3,1);stat = num/sum(num);
        point = (rand(3)>0.5);
        C(kx+p,ky+p) = point*count;
        P(kx+p,ky+p) = point*stat(1);
        S(kx+p,ky+p) = point*stat(2);
        G(kx+p,ky+p) = point*stat(3);
        dot(kx+p,ky+p) = point;
end;
colour = rand(count,3);
[i,j,v] = find(C);
size (i);
figure(gcf);
str = num2str(v);

zoom on;
plot(i,j,'.',...
    'Color','green', ...
    'MarkerSize',7);axis square;
axis([0 m+1 0 m+1]);
text (i,j,str,'FontSize',7);
%neighbors
n = [m 1:m-1];
e = [2:m 1];
s = [2:m 1];
w = [m 1:m-1];


for rounds = 1:iteration
maxn = max(v);
minn = min(v);
```

```
%count through all clones
for inc = minn:maxn;
Cnew = C==inc;
Cnew1 = C.*Cnew;
[in,jn,vn] = find(Cnew1);
prop(rounds,inc) = length(in)/(m*m);
col = colour(inc,:);
figure(gcf);
str = num2str(vn);
plot(in,jn,'.', ...
     'Color',colour(inc,:), ...
     'MarkerSize',15);axis square;
axis([0 m+1 0 m+1]);
%text (in,jn,str,'FontSize',7);
hold on;
end
drawnow
hold off;

%number of neigbors
Nneig = (dot(n,:) + dot(s,:) + dot(:,e) + dot(:,w) + ...
            dot(n,e) + dot(n,w) + dot(s,e) + dot(s,w));

%mean score of neighbors
NP = (P(n,:) + P(s,:) + P(:,e) + P(:,w) + ...
       P(n,e) + P(n,w) + P(s,e) + P(s,w))/8;
NS = (S(n,:) + S(s,:) + S(:,e) + S(:,w) + ...
       S(n,e) + S(n,w) + S(s,e) + S(s,w))/8;
NG = (G(n,:) + G(s,:) + G(:,e) + G(:,w) + ...
       G(n,e) + G(n,w) + G(s,e) + G(s,w))/8;

%sum of loosing neighbors
sP = P>NP;
sS = S>NS;
sG = G>NG;
sc = sP + sS + sG;

%cells that have to be moved according condition
di = cat(3,(sc(n,:)>sc),(sc(:,e)>sc),(sc(s,:)>sc),(sc(:,w)>sc),(sc(n,e)>sc),(sc(n,w)>sc),(sc(s,e)>sc),(sc(s,w)>sc));
m1 = cat(3,C,P,S,G,sc);
ih = 1;
jo = 1;

c1 = randperm(8);


%loop through variables C,P,S,G
for ih = 1:4
            A2 = 0;
            m1n = m1(:,:,ih);
   m2 = cat(3,m1n(n,:),m1n(:,e),m1n(s,:),m1n(:,w),m1n(n,e),m1n(n,w),m1n(s,e),m1n(s,w));
            m2n = m2(:,:,c1(1));
            dn = di(:,:,c1(1));
            tot = dn;
            new1 = (m1n.*(dn==0))+(m2n.*(dn));

   %loop through 8 neighbours
            for jo = 2:8
                        ka = jo-1;
                        c1k = c1(ka);
                        c1j = c1(jo);
                        m2n = m2(:,:,c1j);
                        dn = di(:,:,c1j);
                        dnl = di(:,:,c1k);
                        tot = dnl+A2;
                        A1 = m2n.*dn;
```

```
                    A2 = dn>0&tot==0;
                    A3 = A1.*A2;
                    new1 = (A2==0).*new1+A3;
          end
para(:,:,ih) = new1;
clear A1 A2 A3 tot m2n dn dnl new1 m2 m1n
end
%open gaps at random locations in the meadow
gap = full(matr);
p2 = -gapsize:gapsize;
pmax2 = max(p2);
pmin2 = min(p2);
for count=1:gapfreq;
                    kx2=floor(rand*(m+(-1-pmax2+pmin2))+(1-pmin2)); ky2=floor(rand*(m+(-1-pmax2+pmin2))+(1-pmin2));
          gap(kx2+p2,ky2+p2) = (length(p2)>0.5);%density of disturbance per gap

end;

para(:,:,2).*gap;
gap = gap==0;
%implement the gaps into meadow
C = para(:,:,1).*gap;
P = para(:,:,2).*gap;
S = para(:,:,3).*gap;
G = para(:,:,4).*gap;

[i,j,v] = find(C);

clear gap Cnew Cnew1 in jn vn

end

%plot dynamics of first 10 genets
prop
figure(2);
plot(gen,prop(:,1),'c-',gen,prop(:,2),gen,prop(:,3),'b:p',...
gen,prop(:,4),gen,prop(:,5),gen,prop(:,6),gen,prop(:,7),gen,prop(:,8),gen,prop(:,9),gen,prop(:,10));
axis([1 iteration 0 1]);

%plot size distribution of genets
figure(3);
table = tabul(v)
anew = (sort(table(:,2)));
bnew = anew(length(anew):-1:1);
bar(bnew)
```

CURRICULUM VITAE

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ERKLÄRUNG

Hiermit versichere ich, dass diese Abhandlung – abgesehen von der Beratung durch meine akademischen Lehrer – nach Inhalt und Form meine eigene Arbeit ist und dass ich keine als die angegebenen Hilfsmittel und Quellen verwendet habe. Die Arbeit hat bisher weder ganz noch zum Teil an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegen. Teile dieser Arbeit wurden als Manuskripte bei Zeitschriften eingereicht, mit Thorsten Reusch als Koautor. Kapitel III ist bei Marine Ecology Progress Series im Druck. Kapitel I wurde beim Journal of Evolutionary Biology eingereicht, Kapitel II bei Molecular Ecology und Kapitel IV bei Heredity.

Plön den, 27. Mai 2002 August Hämmerli